

AMPK REGULATES HER2 AND EGFR IN BREAST CANCER

by

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A dissertation submitted to Johns Hopkins University in conformity with the
requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

March 2014

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Abstract

The AMP-activated Protein Kinase (AMPK) is a master regulator of cell metabolism, controlling fatty acid synthesis, protein synthesis and carbohydrate metabolism. Physiological states with increased AMPK activity are associated with decreased cancer incidence, leading to interest in AMPK as a potential target for cancer therapy and prevention. To explore a possible role of AMPK modulation in breast cancer therapy, we investigated how activation of AMPK affects breast cancer cell signaling and survival. Initial experiments found that breast cancer cell lines with amplification and over-expression of HER2 (BT474, HCC1419, and SKBR3) or EGFR (MDA231 and HCC1806) are 2-fold to 5-fold more sensitive to cytotoxic effects of AICAR, a canonical pharmacological activator of AMPK, than breast cancer cell lines that lack HER2 or EGFR overexpression (MCF7 and DU4475).

In parallel to this activation of AMPK, we observed dose- and time-dependent inhibitory effects on phosphorylation and activity of HER2 and EGFR in these AICAR-treated, HER2-amplified breast cancer cells, with activation of AMPK and suppression of HER2/ EGFR activity preceding commitment to cell death. To further explore how AMPK activity affects HER2/ EGFR, we stably transfected HER2-amplified breast cancer cells with constitutively active AMPK α and observed that AMPK activated by this genetic manipulation also leads to decreased HER2 and EGFR phosphorylation and associated downstream signaling as well as reduced cell growth.

Finally, we found that morin, a flavonoid compound previously found to inhibit fatty acid synthesis, also activates AMPK and inhibits HER2 and EGFR signaling in parallel, further supporting the link across these pathways. Metformin, another activator

of AMPK, also inhibited EGFR phosphorylation in HER2-amplified breast cancer. Our results lead us to postulate that AMPK regulates HER2 and EGFR activity in HER2-amplified breast cancer cells, and activation of AMPK might provide therapeutic benefit in such cancers.

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Acknowledgments

First, I would like to express my deepest gratitude to Dr. Edward Gabrielson for his constant guidance, support and endless optimism. I would also like to thank the Gabrielson lab members, past and present, for providing a collegial and welcoming work environment. I would like to especially thank Dr. Juhyung Woo and Ellen Tully for their experimental assistance.

Next, I would like to express my sincere gratitude to Dr. Ben Ho Park, not only for his great advice, but also for his generosity with his time. I would also like to express my profound gratitude to Dr. Bill Isaacs for his scientific advice and his kindness in helping me complete an important segment of my thesis project.

I would like to thank the Pathology Department for giving me the opportunity to complete a doctorate degree at the Johns Hopkins School of Medicine.

I would also like to thank all my friends for their companionship and support during my graduate career.

Finally, I would like to express a special thank you to my family. My deepest appreciation and heartfelt thanks goes out to my father, mother, father-in-law and mother-in-law for their constant love and support. Last, but not least, I would like to thank my husband, Darshil, for his abundance of love, advice and support during graduate school. We did it!

Table of Contents

Abstract.....	ii
Acknowledgments.....	iv
Table of Contents.....	v
List of Figures.....	vii
Chapter 1: Introduction.....	1
1.1 Cancer Background.....	1
<i>1.1.1 Cancer Metabolism</i>	<i>1</i>
<i>1.1.2 Breast Cancer</i>	<i>3</i>
1.2 ErbB Family.....	5
<i>1.2.1 HER2</i>	<i>5</i>
<i>1.2.2 EGFR.....</i>	<i>7</i>
1.3 AMP-activated Protein Kinase (AMPK).....	10
<i>1.3.1 AMPK Structure and Activation</i>	<i>10</i>
<i>1.3.2 AMPK Activators</i>	<i>13</i>
<i>1.3.3 AMPK Downstream Targets</i>	<i>17</i>
<i>1.3.3.1 Metabolism</i>	<i>17</i>
<i>1.3.3.2 Regulation of Mitochondrial Biogenesis and Autophagy.....</i>	<i>18</i>
<i>1.3.3.3 Cell Cycle Regulation.....</i>	<i>19</i>
<i>1.3.3.4 Transcriptional Targets.....</i>	<i>19</i>
<i>1.3.3.5 Cell Polarity.....</i>	<i>20</i>
<i>1.3.3.6 AMPK and Cancer</i>	<i>21</i>
Chapter 2: Experimental Procedures.....	23
Chapter 3: AMPK Regulates HER2 and EGFR Activation.....	28

3.1 Introduction.....	28
3.2 Results.....	29
3.2.1 Activation of AMPK decreases Breast Cancer Cell Viability.....	29
3.2.2 AMPK activation reduces the level of HER2 and EGFR autophosphorylation	31
3.2.3 Genetic studies confirm the role of AMPK in the regulation of HER2 and EGFR activation.....	31
3.2.4 EGFR ligands rescue breast cancer cells from AMPK-based cytotoxicity.....	32
3.2.5 AMPK directly binds HER2 and phosphorylates HER2 and EGFR.....	32
3.2.6 AMPK in vitro kinase assay reveals phosphorylation sites on HER2 and EGFR for AMPK.....	33
3.3 Discussion.....	34
 Chapter 4: AMPK is activated in Breast Cancer Cells by other Compounds, including Morin, a Natural Flavonoid.....	68
4.1 Introduction.....	68
4.2 Results.....	69
4.2.1 AMPK-activating compounds decrease HER2+ breast cancer viability.....	69
4.2.2 Metformin and morin treatment activate AMPK in breast cancer cell lines.....	70
4.2.3 Metformin and morin treatment inhibit the activation of EGFR in HER2+ breast cancer.....	71
4.2.4 Morin and the level of cAMP in breast cancer.....	71
4.3 Discussion.....	73
 References.....	91
 Curriculum vitae.....	116

List of Figures

Figure 1: AICAR reduces the survival of HER2-amplified breast cancer cell lines.....	38
Figure 2: Analysis of HER2 and EGFR levels in selected breast cancer cell lines.....	39
Figure 3: Sensitivity profiles of ER+ and HER2+ breast cancer cell lines to continuous AMPK activation.....	40
Figure 4: Cell growth inhibition in triple negative breast cancer cell lines exposed to continuous AMPK activation.....	41
Figure 5: EGFR overexpression in the MCF10A cell line increases sensitivity to AICAR treatment.....	42
Figure 6: Cell cycle analysis of MCF7 and SKBR3 cells under 24 hour AICAR treatment.....	43
Figure 7: Cell cycle analysis of MCF7 and SKBR3 cells under 48 hour AICAR treatment.....	44
Figure 8: AICAR treatment causes apoptotic cell death in SKBR3 cells.....	45
Figure 9: AICAR treatment activates AMPK in breast cancer cell lines.....	46
Figure 10: AICAR treatment activates AMPK in HER2+ breast cancer cell lines.....	47
Figure 11: AICAR treatment activates AMPK in triple negative breast cancer cell lines.....	48
Figure 12: AICAR treatment activates AMPK in the EGFR overexpressing cell lines MCF10A #7 and #9.....	49
Figure 13: AMPK activation leads to decreased levels of HER2 and EGFR phosphorylation.....	50

Figure 14: AMPK activation leads to decreased levels of phosphorylated HER2, HER3, and EGFR in HER2+ breast cancer cell lines.....	51
Figure 15: AMPK activation leads to decreased EGFR activation.....	52
Figure 16: AMPK activation inhibits EGFR activation in triple negative breast cancer.....	53
Figure 17: Regulatory role of AMPK in HER2 and EGFR activation is confirmed by genetically regulating AMPK levels and activity.....	54
Figure 18: Knockdown of AMPK affects EGFR activation in triple negative breast cancer.....	55
Figure 19: Regulatory role of AMPK in HER2 and EGFR activation is confirmed with AMPK Constitutively Active (CA) and Dominant Negative (DN) constructs in SKBR3.....	56
Figure 20: Regulatory role of AMPK in HER2 and EGFR activation is confirmed with AMPK Constitutively Active (CA) and Dominant Negative (DN) constructs in HCC1419.....	57
Figure 21: Constitutively Active AMPK regulates EGFR phosphorylation.....	58
Figure 22: EGF treatment rescues AICAR-mediated growth inhibition in HCC1419 breast cancer.....	59
Figure 23: EGF treatment rescues AICAR-mediated growth inhibition in BT474 breast cancer.....	60
Figure 24: Identification of an AMPK substrate consensus sequence on HER2.....	61
Figure 25: Activated AMPK phosphorylates an AMPK consensus site on HER2 and EGFR.....	62
Figure 26: AMPK co-immunoprecipitates with HER2 in HER2-amplified breast cancer.....	64

Figure 27: Proposed model of AMPK action on HER2.....	65
Figure 28: Identification of potential AMPK phosphorylation sites on HER2 and EGFR.....	66
Figure 29: Activated AMPK phosphorylates an AMPK consensus site on HER2 and EGFR.....	67
Figure 30: AMPK-activating agents reduce the survival of HER2- amplified breast cancer cell lines.....	76
Figure 31: Cell growth inhibition in HER2+ breast cancer cell lines treated with morin.....	77
Figure 32: Cell growth inhibition in EGFR+ breast cancer cell lines treated with morin.....	78
Figure 33: Morin treatment in ER+ breast cancer does not affect cell viability.....	79
Figure 34: EGFR-dependent cell line, BT549, displays heightened sensitivity to metformin treatment.....	80
Figure 35: Metformin treatment activates AMPK in HER2+ breast cancer.....	81
Figure 36: Morin treatment activates AMPK in HER2+ breast cancer.....	82
Figure 37: Inhibition of FASN does not activate AMPK.....	84
Figure 38: Metformin treatment inhibits EGFR activation.....	85
Figure 39: Morin treatment inhibits EGFR activation in HER2+ breast cancer.....	86
Figure 40: Morin does not inhibit ERK1/2 signaling.....	87
Figure 41: Treatment of metformin or C75 inhibit mTOR activation.....	88

Figure 42: Measurement of cAMP levels in BT474 following pharmacological treatment.....	89
Figure 43: cAMP measurement in HCC1419 breast cancer cells following pharmacological treatment.....	90

Chapter 1: Introduction

1.1 Cancer Background

In 2002, nearly 25 million people worldwide were estimated to be living with cancer [1]. While the most common causes of cancer-related death are due to lung, stomach, or liver cancer, breast cancer remains the most prevalent form of cancer in the world [2].

The term cancer does not refer to a single disease, but rather a group of diseases that is characterized with some common attributes [3]. Cancer cells are distinguished by the following characteristics: the ability to evade cell death, to maintain proliferative signaling, to induce angiogenesis, to evade growth suppressors, to metastasize and to retain replicative immortality [4]. Tumorigenesis is considered a multistep process, with oncogene activation, loss of tumor suppressor activity and induction of aneuploidy all important for cancer development [3, 5].

1.1.1 Cancer Metabolism

In the 1920s, Otto Warburg first observed the widespread abnormal features of cancer cell metabolism [6, 7]. In the presence of oxygen, normal cells typically rely on oxidative phosphorylation for energy production. Contrastingly, in anaerobic conditions, cells undergo glycolysis. However, cancer cells undergo “aerobic glycolysis,” which is defined as glycolysis in the presence of oxygen, now known as the Warburg Effect [6-8]. Compared to oxidative phosphorylation, glycolysis has approximately an 18-fold lower ATP production efficiency [4]. To account for this lower level of energy production, cancer cells up-regulate glucose transporters and accordingly display a heightened dependence on glucose [8-10]. Dependence on aerobic glycolysis has several benefits for

cancer cells. First, if the rate of aerobic glycolysis is high enough, ATP production can surpass that of oxidative phosphorylation [8]. Second, glycolysis produces intermediate compounds needed for biosynthetic pathways [8]. For example, ribose sugars are used for nucleotides and glycerol and citrate for lipids [8].

Many oncogenes commonly mutated, or activated in cancer, control tumor cell metabolism [11]. The PI3K/AKT/mTOR pathway is a highly conserved system that cells utilize to respond to growth factors [8, 12]. Activation of the PI3K/AKT/mTOR pathway supports increased biosynthesis and increased transport of glucose and other nutrients [8, 13]. AKT increases glycolysis and lactate production, while mTOR increases protein synthesis [8, 14-16]. The RAS oncogene, a GTP-binding protein, also has a role in promoting glycolysis [9, 17]. c-Myc, an oncogenic transcription factor, is often overexpressed in cancer [9, 17, 18]. c-Myc, thought to be overexpressed in approximately 70% of human tumors, upregulates the expression of various metabolic genes, including *pyruvate kinase* and *lactate dehydrogenase (LDH-A)* [17, 18].

Inhibition of lactate dehydrogenase (LDH-A) has been shown to prevent the Warburg Effect and essentially reverts cellular energy production to oxidative phosphorylation [19, 20]. Blocking aerobic glycolysis in tumor cells leads to attenuated tumor growth, which suggests that aerobic glycolysis is necessary for tumor cell survival and growth [9]. Several tumors also demonstrate increased endogenous fatty acid synthesis [21-25], and in these tumors, high levels of fatty acid production are necessary to fuel membrane production and lipid-based post-translational modifications of proteins [21, 25]. Fatty acid synthase (FASN), a 250-270 kDa homodimeric protein, synthesizes long chain fatty acids by using acetyl-CoA as a primer, malonyl-CoA as a 2-carbon

donor, and NADPH as a reducing equivalent [21, 26, 27]. Acetyl-CoA Carboxylase 1 (ACC1) and Acetyl-CoA Carboxylase 2 (ACC2) regulate fatty acid synthesis by controlling the levels of malonyl-CoA [28]. High levels of FASN have been observed in several different types of cancer, including breast, ovary, lung, melanoma, prostate, endometrium, colon and thyroid [25, 29]. FASN is expressed at low or undetectable levels in most normal adult tissues, with exceptions being adult liver and adipose tissue [29]. FASN expression is highest in metastatic tumors, and tumor metastases and correlates with decreased survival and disease recurrence [29]. Inhibition of tumor FASN *in vitro* leads to tumor cell cycle arrest and apoptosis [30]. FASN expression has been shown to be stimulated by Epidermal Growth Factor Receptor (EGFR) and Human Epidermal growth factor Receptor 2 (HER2) signaling [21, 31-34]. In fact, high expression of HER2 is correlated with heightened sensitivity to pharmacological inhibition of FASN [33, 34].

1.1.2 Breast Cancer

Breast cancer is the most common type of cancer in women in the United States, and the second most common cause of death from cancer in women [35]. In 2010, approximately 41,000 women died from breast cancer-related conditions [35]. Risk factors for breast cancer include: family history, obesity, alcoholism, nulliparity, late age of first pregnancy, late menopause, and exposure to radiation [36, 37]. Breast cancer is a broad term that covers a heterogeneous group of diseases [38]. Premalignant lesions that have increased risk of malignant transformation include atypical ductal hyperplasia and hyperplastic alveolar nodules [36]. Earlier, with immunohistochemistry (IHC), scientists determined that 3 major subtypes of breast cancer existed: hormone receptor-positive,

HER2-positive, and “triple negative” cancers, which lack expression of hormone receptors as well as HER2 [38]. Estrogen Receptor (ER) and Progesterone Receptor (PR) positive tumors are known to account for approximately 60% of breast cancers. Contrastingly, HER2 positive breast cancer accounts for approximately 25% of breast cancers [36]. About 15% of breast cancers lack expression of ER, PR, and HER2 and are classified as triple negative breast cancer (TNBC) [36]. Compared to other subtypes of breast cancer, TNBC is characteristically more aggressive, with an increased likelihood of distant recurrence and death [39, 40].

Based on molecular profiling, breast cancer can be divided into at least seven different biologic subtypes: luminal A, luminal B, luminal C, HER2-positive, basal-like, claudin-low, and normal breast-like [38]. Luminal breast cancers are named for their similarity to normal luminal breast epithelium [38]. Luminal A tumors, about 40% of all breast cancers, have the best prognosis and are characterized by high expression of ER-related genes, low expression of HER2-related and proliferation-related genes [36]. Luminal B tumors are less common and are characterized by lower expression of ER-related genes, variable expression of HER2-clusters, and higher expression of proliferation-related genes [36]. Luminal C is distinguished by its high expression of a novel set of genes with unknown function. Luminal B and C tumors have a worse relapse-free survival and overall survival, as compared to luminal A tumors [36]. The HER2-positive subtype is characterized by high expression of HER2-related and proliferation genes [36]. Basal-like tumors are classified based on their similar expression pattern to basal epithelial cells. These tumors are ER/PR and HER2-negative (triple negative) and display wide genomic instability [36]. BRCA1 mutations are strongly

associated with basal-like breast cancer [36]. Basal-like breast cancer is also characterized by high expression of EGFR, p-cadherin, smooth muscle actin, c-kit, and cytokeratins 5, 6, 14, and 17 [36]. The claudin-low subtype is characterized by low expression of luminal differentiation markers, and high expression of epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features [36]. Most claudin-low tumors tend to be HER2 and ER/PR negative (triple negative). Normal breast-like tumors cannot be characterized into either of the other subtypes. Normal breast-like tumors demonstrate high expression of genes known to be expressed in adipose tissue, as well as high expression of basal epithelial genes [36].

1.2 ErbB Family

HER2, an oncogene important in many breast cancers, is a member of the ErbB family of receptor tyrosine kinases. The ErbB family is composed of 4 homologous proteins: EGFR (HER1), HER2, HER3, and HER4. EGFR, HER2 and HER3 have all been implicated in promoting tumorigenesis [37]. As receptor tyrosine kinases (RTK), these transmembrane proteins influence cell growth, differentiation, and survival [37]. RTKs contain 3 domains: an extracellular domain important for ligand binding, a transmembrane domain that anchors the protein on the cell membrane, and an intracellular domain that contains the kinase segment of the protein [37]. Ligand binding induces hetero- or homodimerization of the ErbB family of kinases, followed by autophosphorylation of key activating residues on the intracellular domain of the RTK [37, 41]. Autophosphorylation of the RTK leads to recruitment of signaling molecules and activation of a variety of downstream signaling pathways [41].

1.2.1 HER2

HER2 is an 185 kDa transmembrane oncoprotein that occurs in 20-25% of all breast cancers [41]. HER2 overexpression and/or amplification have also been observed in colon, bladder, ovarian, endometrial, lung, uterine cervix, head and neck, gastric and esophageal carcinomas [42-48]. Unlike the other ErbB family members, HER2 is an “orphan receptor,” meaning it has no known ligand [37, 41]. HER2 is the preferred binding partner for the other members of the ErbB family. HER2 is considered “constitutively active,” as it requires no binding of a ligand to be active [41]. HER2-containing heterodimers evade normal inactivation processes by internalizing relatively slowly and by returning to the cell surface- thereby avoiding the process of degradation [49]. HER2 overexpression is associated with anchorage-independent cancer cell growth, increased cell migration, increased angiogenesis and increased cell proliferation [49]. HER2 downstream signaling is important for cancer growth and development. In cancer, the Ras/Raf/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways are two important signaling cascades that promote cancer growth and survival downstream of HER2 signaling [41, 49].

Current therapeutic options for patients with HER2-positive tumors include trastuzumab (Herceptin®), lapatinib (Tykerb®), and pertuzumab (Perjeta®) [41]. Trastuzumab, a humanized monoclonal antibody that binds to HER2, was approved for use in metastatic breast cancer in 1998 and early stage breast cancer in 2008 [41]. Recent clinical trials have been exploring the use of trastuzumab in HER2-positive gastric or gastric-esophageal carcinomas [50]. Although the exact mechanism of action is unknown, trastuzumab binds to the juxtamembrane portion of the extracellular domain of HER2 [41]. It has been proposed that trastuzumab may induce apoptosis, inhibit angiogenesis,

inhibit cell signaling and cause antibody-dependent cell cytotoxicity [36, 51]. A phase III study of trastuzumab plus various chemotherapy regimens showed a significant improvement in overall survival (25.1 vs. 20.3 months) and overall response rate (50 vs. 32%) in breast cancer patients [52]. However, approximately half of HER2-positive breast cancer patients display resistance to trastuzumab and of the patients who respond, most end up with progressive disease while on trastuzumab therapy [41, 51]. In the adjuvant setting, addition of trastuzumab to the standard chemotherapy regimen reduced rates of reoccurrence by over 50% [53]. Trastuzumab resistance has been correlated to activation of both the PI3K and the Insulin-like growth factor receptor (IGF-IR) signaling pathways [54-56]. High levels of ErbB family ligands (growth factors) can also drive resistance to RTK inhibitors [57]. Lapatinib, a small molecule inhibitor of both EGFR and HER2 tyrosine kinase activity, was approved for use in HER2-positive breast cancer in 2007 [41]. Phase I studies of lapatinib have demonstrated response both as a single agent (6%) and in combination with trastuzumab (26%) [37, 58]. Pertuzumab, a recombinant humanized monoclonal antibody, functions by disrupting HER2 dimerization [41]. Pertuzumab was approved by the FDA in 2012 for patients with metastatic HER2-positive breast cancer [59]. Patients receiving pertuzumab in combination with docetaxel and trastuzumab displayed increased progression-free survival as compared to the control group (18.5 vs. 12.4 months) [59]. Similar to trastuzumab, pertuzumab stimulates antibody-dependent, cell-mediated cytotoxicity [60]. Contrastingly, pertuzumab binds to HER2 and prevents its dimerization with HER3, thereby preventing HER2 downstream signaling [41].

1.2.2 EGFR

EGFR – also known as HER1, is a 170 kDa protein that overexpressed or overactive in a variety of solid tumor types, including about 20% of breast cancer [56, 61]. EGFR overexpression is more often found in triple negative breast cancer and is associated with a more aggressive phenotype [56]. However, EGFR inhibition to date has relatively limited clinical benefit in breast cancer patients [56, 62]. Elevated EGFR levels have a strong correlation with poor prognosis in head and neck, glioma, cervical, ovarian, esophageal and bladder cancers [62]. In gastric, breast, endometrial, and colorectal cancers, EGFR overexpression has modest prognostic implications, while in non-small cell lung carcinoma, EGFR overexpression seldom has prognostic implications [62].

Eleven ligands are known to bind to the ErbB family of tyrosine kinase receptors [61]. Ligands that specifically bind to EGFR are: epidermal growth factor (EGF), transforming growth factor- α (TGF- α), amphiregulin, and epigen [61]. Ligands that bind to both EGFR and HER4 include: betacellulin, heparin-binding EGF, and epiregulin [61]. Neuregulin 1 (NRG1) and 2 (NRG 2) bind both HER3 and HER4, while NRG3 and NRG4 only bind HER4 [61].

Mutations of EGFR are quite commonly seen in cancer. EGFR VIII is a deletion mutation of amino acids 30-297, resulting in a EGFR protein that is constitutively activated without requiring ligand binding [61]. EGFR VIII is reported to occur in 30-50% of glioblastomas, 21% of squamous head and neck carcinomas, 5% of squamous lung carcinomas, and 5% of breast carcinomas [61, 62]. EGFR VIII is associated with poor survival in glioblastoma patients; median survival of patients with EGFR VIII mutation is 45 weeks, as compared to 85 weeks in the non-EGFR VIII patient population [63]. Other mutations of EGFR include mutations that involve the kinase domain.

Mutations in exon 18, the nucleotide binding loop region, account for 5% of EGFR mutations, while mutations in exon 19, account for 45% of EGFR mutations [62]. The L858R substitution in exon 21 accounts for another 40-45% of EGFR mutations. EGFR exon 20 mutations account for up to 4% of all EGFR mutations, with T790M comprising about 50% of exon 20 mutations [62]. The T790M mutation is correlated to increased resistance to tyrosine kinase inhibitors (TKI) [62]. About 60% of patients with acquired resistance to EGFR TKI have tumors that are characterized with the T790M mutation [64]. While mutations of EGFR are rare in breast cancer, the frequency of activating mutations of EGFR in hereditary breast cancer is greater than in spontaneous breast cancer [65].

While EGFR inhibitors have been tested in clinical trials for the treatment of breast cancer, the results of such trials have not been encouraging [65]. For example, clinical trials with the tyrosine kinase inhibitors gefitinib and erlotinib failed to demonstrate an improved response rate. However, this poor outcome may relate to the fact that these studies did not select patients that had established EGFR expression [65]. Furthermore, a phase II clinical trial determined the overall response rate of cisplatin with cetuximab (a chimeric EGFR-targeting monoclonal antibody) was doubled to that of cisplatin alone (20 vs. 10.3%) [65]. Panitumumab, a humanized monoclonal antibody specific to EGFR, demonstrated an 80% overall response rate in combination with 5-fluorouracil, epirubicin (epidoxorubicin), and cyclophosphamide followed by docetaxel for neoadjuvant therapy in TNBC patients [65]. As antibodies, cetuximab and panitumumab function by blocking the binding of ligands to the extracellular domain of

EGFR, promoting receptor internalization (and subsequent degradation), and mediating antibody- and complement-mediated cytotoxicity [64].

1.3 AMP-activated Protein Kinase (AMPK)

Cellular energy balance is vital part of all cell biological processes, including cell growth, division and movement. In cells, ATP levels are maintained in the low millimolar range, which indicate a delicate balance between energy-consumption processes and energy-producing processes [66]. AMP-activated protein kinase (AMPK) is a highly conserved cellular sensor of AMP and ADP levels [66-68]. AMPK orthologues have been found in *Arabidopsis*, *Saccharomyces cerevisiae*, *Dictyostelium*, *Caenorhabditis elegans*, *Drosophila*, and *Physcomitrella patens* [67].

1.3.1 AMPK Structure and Activation

AMPK is a heterotrimeric serine/threonine kinase composed of α , β , and γ subunits [66-68]. In humans, two isoforms of the α subunit ($\alpha 1$ and $\alpha 2$), two isoforms of the β subunit ($\beta 1$ and $\beta 2$), and three isoforms of the γ subunit ($\gamma 1$, $\gamma 2$, and $\gamma 3$) exist [66]. Twelve different combinations of AMPK trimers can exist. Some AMPK combinations are not found in specific tissues and each trimer combination displays a distinct spectrum of biochemical properties [66]. The $\alpha 1$ isoform is predominantly found in white adipose tissue, blood cells, smooth muscle, endothelial cells and nerve [66], whereas $\alpha 2$ is the predominant isoform in muscle and heart [66]. While AMPK proteins are generally found in the cytoplasm, several AMPK subunits have also been found in the nucleus ($\alpha 2$, $\beta 2$, $\gamma 1$, and $\gamma 3$), suggesting a possible role in the regulation of gene expression [66].

The α subunit of AMPK represents the catalytic region of the protein, containing the key threonine 172 residue, whose phosphorylation is required for complete AMPK

enzymatic activity [66]. The α subunit partners with the β and γ subunits through its C-terminal domain [66]. The β subunits contain a carbohydrate-binding domain, which allows AMPK to interact with glycogen particles. The γ subunit contains four tandem repeats known as cystathionine β -synthase (CBS) motifs, which allows AMPK to bind two AMP, ADP, or ATP molecules in a mutually exclusive way, and a third AMP molecule in a non-exchangeable way [66, 69]. While binding of ATP to AMPK maintains the kinase in a low activity state, binding of AMP to AMPK leads to a fivefold activation of the enzyme [66]. AMP binding also makes AMPK an inferior substrate for α subunit threonine 172 phosphatase, which allows for elevated levels of phosphorylated AMPK α 172 [66, 68, 69]. The allosteric effects of AMP binding also favor AMPK α phosphorylation by upstream AMPK-activating kinases. AMP binding to AMPK and phosphorylation of AMPK α at threonine 172 result in a greater than 1,000-fold activation of AMPK [66, 68].

In humans, six different point mutations in the gene encoding the γ 2 subunit are responsible for the Wolff-Parkinson-White syndrome (WPWS), a hereditary heart disease that is usually associated with hypertrophic cardiomyopathy (HCM) [68]. The mechanism behind how these mutations cause WPWS and HCM is currently unknown, but preliminary evidence suggests that these mutations work to increase the basal activity of AMPK [68].

Identified upstream kinases that phosphorylate AMPK α 172 are: 1) liver kinase B1 (LKB1), calcium and calmodulin-dependent protein kinase kinase (CaMKK), 2) TGF- β -activating kinase (TAK1), and 3) the protein of the ataxia-telangiectasia mutated (ATM) gene [66, 70]. In hypothalamic neurons, T cells and endothelial cells, CaMKK

seems to play a role in the activation of AMPK [71]. LKB1, also known as serine/threonine kinase 11 (STK11) is ubiquitously expressed and is the main kinase required for AMPK activation [70]. LKB1 functions in a heterotrimeric complex with Sterile-20-Related Adaptor (STRAD) and Mouse protein 25 (Mo25) [66]. Most evidence supports the concept that LKB1 is a constitutively active kinase [66]. LKB1 is the main upstream kinase for the 13 members of the AMPK-related kinase family [66]. LKB1 is a known tumor suppressor and loss of function mutations result in Peutz-Jeghers syndrome [66, 70], which is characterized by multiple hamartomatous polyps in the gastrointestinal tract and a heightened risk of gastrointestinal adenocarcinomas [70]. Somatic mutations of LKB1 have been observed in several types of cancer, including: lung adenocarcinomas (34%), cervical carcinomas (20%), and squamous cell carcinomas (19%) [70]. Low expression of LKB1 has been reported in clear cell renal carcinoma, resulting in lower AMPK activity and a growth advantage of cancer cells *in vitro* and *in vivo* [72]. Complete ablation of LKB1 in the mouse model leads to embryonic lethality, but heterozygous deletion increases the incidence of spontaneous tumors in the intestine and stomach [70]. A hypomorphic mutation that decreases LKB1 activity rapidly increases tumor development in PTEN^{+/-} mice; however, treatment with AMPK pharmacological activators significantly delay tumor development [70]. As LKB1 is frequently co-mutated with KRAS in non-small cell lung cancer (NSCLC), a mouse model bearing a conditionally activated allele of *Kras* and a conditionally inactivated allele of *Lkb1* had a phenotype of increased tumor incidence, metastasis and acceleration of death [73]. LKB1 is reportedly phosphorylated by ERK and Rsk, which inhibit LKB1 binding and phosphorylation of AMPK [74].

1.3.2 AMPK Activators

As a cellular energy sensor, AMPK is activated by metabolic stresses that either inhibit ATP synthesis or accelerate ATP consumption [69]. AMPK can be activated by metabolic stresses that inhibit ATP production via oxidative phosphorylation, such as arsenite, oligomycin, antimycin A, azide, dinitrophenol, hydrogen peroxide, hypoxia or ischemia [75-80]. AMPK is also activated in skeletal muscle in response to exercise (contraction of muscle fibers) [81-83]. AMPK is also regulated by physiological variations in glucose; most notably, AMPK is activated in conditions of low glucose [84, 85]. In humans, activation of AMPK in the hypothalamus leads to increased feeding behavior, indicating a conserved role of AMPK in maintaining energy homeostasis [86, 87]. The gut hormone ghrelin and the cannabinoids have been reported to activate AMPK in the hypothalamus [87, 88]. Leptin, an adipokine (cytokine released by adipocytes), represents a signal that food stores are adequate [89]. Leptin was reported to activate AMPK in the mouse muscle, yet inhibit AMPK activity in the hypothalamus [86, 90]. AMPK is also activated by adiponectin (ACRP30) in skeletal muscle [91]. Liver AMPK is necessary for adiponectin-dependent lowering of blood glucose [92].

Pharmacological activators of AMPK are commonly utilized for research purposes. The canonical activator of AMPK, AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside), is an adenosine analog that is taken up into the cell by adenosine transporters [75, 93] and then converted by adenosine kinase to the phosphorylated nucleotide ZMP (AICAR monophosphate) [67]. ZMP mimics AMP, binding directly to the AMPK γ subunit and activating AMPK directly [67]. AICAR is not approved for use

in the clinic; however, a clinical trial examining the potential benefit of AICAR for treatment of chronic lymphocytic leukemia (CLL) is currently underway [94].

Metformin and other biguanides (including phenformin) activate AMPK through an indirect mechanism, by blocking ATP production through the inhibition of mitochondrial Complex I of the respiratory chain [95-97]. Metformin, commonly used to treat type 2 diabetes, increases insulin sensitivity and improves glycemic control [98-100]. Early reports indicated that type 2 diabetic patients receiving metformin had a 30% decrease in the incidence of cancer, as compared to patients not receiving metformin [101, 102]. Each five-year metformin treatment period was correlated to a 0.73 reduction in cancer-related death [103]. Metformin and thiazolidinediones, both used for the treatment of diabetes, activate AMPK. A study analyzing the survival of diabetic women with HER2+ cancer determined that metformin or thiazolidinediones use was correlated with decreased breast cancer-related mortality, indicating a link between AMPK activation and inhibition of breast cancer growth [104]. Metformin use in diabetic breast cancer patients was associated with 24% change in the pathologic complete response (pCR) rate, while diabetic cancer patients not using metformin had a 8% pCR rate [105]. Metformin treatment in breast cancer cell lines has been associated with cell cycle arrest, reduction in cyclin D1 and E2F1 [106].

Pemetrexed, a chemotherapeutic drug used for mesothelioma and NSCLC treatment, is another agent that might act in part by activating AMPK. Pemetrexed is well known to inhibit thymidylate synthase, and pemetrexed also inhibits AICART (aminoimidazolecarboxamide ribonucleotide formyltransferase) [107], a folate-dependent

enzyme of purine biosynthesis, which results in increased intracellular ZMP and activation of AMPK [107].

A number of naturally occurring compounds have been reported to activate AMPK. For example, resveratrol, a polyphenol, inhibits F1F0 mitochondrial ATP synthase, which results in an increased AMP to ATP ratio, leading to activation of AMPK [96]. Resveratrol treatment in mice increases metabolic activity, insulin sensitivity, glucose tolerance, mitochondrial biogenesis, and physical endurance [108, 109]. In AMPK α 1^{-/-} knockout mice, resveratrol treatment failed to improve metabolic activity, insulin sensitivity, glucose tolerance, mitochondrial biogenesis, and physical endurance [108, 109], consistent with this activity being due to activation of AMPK. Resveratrol increases the NAD-to-NADH ratio in an AMPK-dependent manner, which is proposed to explain how resveratrol activates Sirt1 [108, 110]. Polyphenol treatment, including resveratrol, apigenin, and S17834, in HepG2 cells not only activated AMPK with 200 fold the potency of metformin, but also decreased lipid accumulation in cells exposed to high levels of glucose [111]. Resveratrol has also been reported to directly inhibit cAMP-specific phosphodiesterases (PDE) [112]. Resveratrol treatment in chronic myelogenous leukemia (CML) not only activates AMPK, leading to downstream inhibition of the mTOR pathway, but also induces autophagy through JNK-dependent accumulation of p62 [113].

Several flavonoid compounds have also been reported to activate AMPK [114-120]. The flavonoids genistein, kaempferol, quercetin, and fisetin have been reported to activate AMPK, although the mechanism of AMPK activation has yet to be elucidated.

Morin, [2-(2',4'-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one], a flavonoid identified in fruits, vegetables, tea and wine [121], has not previously been reported to activate AMPK. Morin is a yellow pigment isolated from the plants of the Moraceae family [122] that has been reported to have beneficial medicinal properties, however. For example, morin has been reported to have anti-inflammatory, anti-neoplastic, and cardioprotective activities [123, 124], and studies have suggested that adequate intake of flavonoid-rich foods (although not specifically morin) may reduce the risk of cancer and coronary heart disease [121, 125, 126]. In laboratory experiments, morin has been shown to have deleterious effects on breast cancer cell lines through disruption of the mitochondrial membrane potential [121]. Morin has been reported to inhibit the growth of COLO205 cells in nude mice as well as protect against chemically induced rat tongue carcinogenesis [127, 128]. Additionally, morin has been attributed with suppressing the proliferation of squamous cell carcinoma, leukemia, and colon cancer cell lines [129-131]. Morin has been shown to induce p21 and activate caspases, suppress AKT activation, induce stress kinase activation, abolish peroxisome proliferator-activated receptor activity, inhibit P-glycoprotein, inhibit lipooxygenase-1, suppress nuclear factor- κ B (NF κ B) activity, suppress inducible nitric oxide synthase and cyclooxygenase-2 (COX-2) expression and inhibit the release of inflammatory cytokines, such as IL-6, IL-8 and tumor necrosis factor (TNF) [127, 128, 131-136]. While morin has not yet reported to activate AMPK, morin has been reported to inhibit fatty acid synthase (FASN) at an *in vitro* IC₅₀ value of 2.33 μ M [137]. Given that AMPK activation also leads to inhibition of FASN (through inhibition of ACC), we explored the possibility that

morin-based inhibition of FASN may function through the activation of AMPK in our work described below.

1.3.3 AMPK Downstream Targets

1.3.3.1 Metabolism

A major target of AMPK activity is fatty acid and cholesterol synthesis. AMPK was first identified as a kinase that phosphorylates and inhibits acetyl CoA carboxylase (ACC) and HMG-CoA reductase, both rate-limiting enzymes in the synthesis of fatty acid and cholesterol, respectively [70]. HMG-CoA reductase inhibitors, statins, have been proposed to prevent cancer in experimental models [138, 139]. As mentioned earlier, FASN and ACC are often highly expressed in several types of cancer [25, 140]. Increased levels of FASN and ACC are attributed to increased expression of the transcription factors, sterol regulatory element binding protein 1 and 2 (SREBP-1 and SREBP-2) [141]. Inhibition of FASN in human ovarian cancer cells leads to activation of AMPK, followed by induction of cytotoxicity. Compound C, a pharmacological inhibitor of AMPK, reportedly prevents the cytotoxic effect of FASN inhibition, suggesting that AMPK activation is necessary for the induction of cytotoxicity by FASN inhibitors [142]. AMPK also stimulates fatty acid uptake through the translocation of the fatty acid transporter FAT/CD36 [143]. AMPK functions to inhibit triglyceride and phospholipid synthesis by inactivating glycerol phosphate acyl transferase, glycogen synthesis by phosphorylation of glycogen synthase, and ribosomal RNA synthesis by phosphorylation of the RNA polymerase I transcription factor TIF-1A (RRN3) [144-146].

AMPK also regulates glucose uptake through the activation of the glucose transporters, GLUT1 and GLUT4 [147, 148]. AMPK phosphorylates histone deacetylase-

5, which increases GLUT4 transcription [149], and AMPK stimulates glycolysis by phosphorylating two of the four isoforms of 6-phosphofructo-2-kinase, the enzyme responsible for the synthesis of the glycolytic activator fructose-2,6-bisphosphate [76, 150]. AMPK also represses transcription of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase mRNAs, thereby inhibiting gluconeogenesis [144, 151].

In addition to these effects on carbohydrate metabolism, AMPK also regulates protein synthesis through a variety of downstream effectors. For example, AMPK inhibits elongation of translation by promoting phosphorylation of eukaryotic elongation factor 2 (eEF2) [152, 153]. Another important target of AMPK is the mammalian target of rapamycin (mTOR) complex-1 (mTORC1); AMPK directly phosphorylates and activates the mTORC1 upstream regulator Tuberous Sclerosis Complex (TSC2) and this activation of TSC2 leads to inhibition of mTORC1. Additionally, AMPK phosphorylates the mTORC1 subunit Raptor, which also functions to inhibit mTORC1 function [154, 155]. As mTORC1 positively regulates cell growth and proliferation by promoting protein, lipid and organelle biosynthesis, AMPK-based inhibition of mTORC1 effectively downregulates several anabolic pathways required for cell growth [156].

1.3.3.2 Regulation of Mitochondrial Biogenesis and Autophagy

Chronic activation of AMPK in the muscle of mice leads to the up-regulation of nuclear-encoded mitochondrial genes and improves the endurance of mice in treadmill running tests [157]. This interesting consequence of AMPK activation is proposed to be due to upregulation of mitochondrial function by enhancing the transcription of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α), a

transcription factor responsible for the biogenesis of new mitochondria as well as the expression of nuclear-encoded mitochondrial genes [144, 158].

AMPK also has a direct effect on the process of autophagy, the process of recycling cytoplasmic components through engulfment by autophagic vacuoles that then fuse with lysosomes [144]. Autophagy is particularly important under conditions of nutrient deprivation as a means to produce catabolic fuels and recycle amino acids for the production of important proteins [144]. The unc-51 like kinase 1 and 2 (ULK1 and ULK2) isoforms initiate the autophagy cascade [144], and ULK1 and ULK2 were found to form a stable complex with AMPK, allowing AMPK to phosphorylate ULK1 at several sites [159-161]. Cells lacking ULK1 or AMPK displayed a marked increase of accumulated mitochondria [160]. In cells where the endogenous ULK1 was replaced by a mutant in which the four serine residues identified as AMPK sites were mutated to alanine, starvation induced the accumulation of abnormal mitochondria [160]. Notably, without AMPK or ULK1 function, fewer cells survived starvation conditions [160].

1.3.3.3 Cell Cycle Regulation

Activation of AMPK in cultured tumor cells causes a G1–S-phase cell cycle arrest that involves the upregulation and stabilization of p53 and the cyclin-dependent kinase inhibitors p21^{Waf1/Cip1} and p27^{Kip1} [144, 162-164]. AMPK is proposed to phosphorylate p53 directly at serine 15 and p27^{Kip1} at threonine 198 [163, 164]. AMPK activation also reduces the cytoplasmic to nuclear ratio of the RNA-binding protein HuR, reducing its ability to stabilize mRNAs involved in cell cycle regulation [75]. Increased AMPK activity also appears to promote cell senescence [75].

1.3.3.4 Transcriptional Targets

AMPK has been reported to phosphorylate and regulate a number of transcription factors and coactivators, including the acetyltransferase p300, some histone deacetylases, and histones [67]. Histone H2B serine 36 is proposed to be an AMPK phosphorylation site, however, this site does not conform well to the AMPK consensus sequence [67]. AMPK has also been linked to regulation of the circadian clock, by phosphorylation of Cry1 serine 71, which targets the protein for ubiquitin-mediated degradation [165]. AMPK also phosphorylates proteins of the CRTC (cyclic AMP-regulated transcriptional co-activators) family, which are transcriptional co-activators for CREB (cAMP response element binding protein) [67]. Additional targets of AMPK include the nuclear receptors HNF4 α (hepatocyte nuclear factor-4 alpha), TR4 (testicular receptor 4), and the zinc-finger protein AREBP (AICAR response element binding protein) [166-168]. The class IIa family of histone deacetylases (HDACs), which represent yet another group of AMPK targets [67], are inhibited by AMPK-mediated phosphorylation [67]. In certain cell types, AMPK is reported to stimulate a set of FOXO-dependent target genes under stress conditions through the direct phosphorylation of FOXO3 and FOXO4 [169]. In addition to phosphorylating transcriptional regulators, AMPK appears to regulate the activity of the deacetylase SIRT1 through effects on NAD⁺ levels [170, 171].

1.3.3.5 Cell Polarity

Embryos lacking LKB1 or AMPK display similar defects in epithelial cell polarity during development [172]. LKB1 appears necessary for the establishment of cell polarity under all conditions, while AMPK is only necessary for the establishment of cell polarity under starvation conditions [173]. Although AMPK-null mice survive and do not seem to have similar effects as *Drosophila* in cell polarity, there still remains evidence

that the LKB1-AMPK pathway is important in maintaining epithelial cell polarity in mammals [144]. Experiments using the intestinal epithelial cancer cell line LS174T, which does not express LKB1, found that re-expression of LKB1 and STRAD caused a rapid remodeling of the actin cytoskeleton and a reformation of an apical brush border membrane [174]. Other experiments using Madin Darby canine kidney (MDCK) epithelial cells found that the removal of Ca^{2+} from the medium results in a loss of polarity and of tight junctions, while restoration of Ca^{2+} in the media re-establishes both polarity and tight junctions, concomitant with activation of AMPK [175]. In Vero cells, AMPK was shown to phosphorylate the microtubule plus-end protein CLIP-170 (Cytoplasmic linker protein 170). Mutation of the AMPK phosphorylation site on CLIP-170 resulted in slower microtubule assembly [176]. AMPK also may play a role in polarizing neurons through the control of phosphatidylinositol 3-kinase (PI3K) localization. By phosphorylating kinesin light chain 2 (KLC2), AMPK was shown to inhibit axonal growth by preventing PI3K localization to the axonal tip [177].

1.3.3.6 AMPK and Cancer

The discovery that LKB1, a known tumor suppressor, was the upstream activating kinase to AMPK provided a key link between AMPK and cancer [178]. AMPK appears to be the main mediator of the LKB1 tumor suppressor effects, as of the AMPK and AMPK-related kinases, only AMPK is known to cause inhibition of cell growth and biosynthesis [144]. The observation that diabetics treated with metformin had a lower incidence of cancer than diabetics not treated with metformin further established the idea that AMPK activation may have anti-tumor effects [101]. Furthermore, treatment of a tumor-prone mouse model with three different activators of AMPK: metformin,

phenformin, or A-769662 (a direct activator of AMPK developed by Abbot) significantly delayed tumor development [179, 180].

Other evidence also suggests that AMPK signaling is important in the development of specific types of cancers. In primary human breast cancer, reduced AMPK signaling was observed in 89.4% and 91.9% in two cohorts of patients [181]. A role for reduced AMPK signaling in melanoma has been suggested to occur through the mutated form of the proto-oncogene B-Raf (V600E, causing constitutive activation) [74], which occurs in up to 50% of all malignant melanomas [144]. In melanoma cell lines with this mutation, LKB1 was found to be phosphorylated at two C-terminal sites by B-Raf downstream kinases [74], and it is highly possible that these phosphorylations on LKB1 inhibit LKB1-dependent phosphorylation and activation of AMPK [74]. Akt-mediated phosphorylation of AMPK at $\alpha 1$ subunit serine 485 ($\alpha 2$ serine 491) has also been proposed to inhibit LKB1-mediated phosphorylation at α subunit threonine 172, thereby inhibiting AMPK activation in cancer [182]. Furthermore, mRNA levels of AMPK $\alpha 2$ inversely correlate with clinical prognosis in ovarian and breast cancers, and are diminished in cancer cells by the activation of the PI3K pathway [183]. In conclusion, while cancer cells appear to need AMPK for survival in conditions of deprivation, cancer cells must also maintain a tight control over AMPK activation [70]. Activation of AMPK must be strictly regulated to prevent inhibition of cellular growth pathways. Therefore, AMPK presents an attractive therapeutic target for the treatment of cancer [70].

Chapter 2: Experimental Procedures

Materials

Anti-phospho-AMPK α (172), phospho-HER2 (1221/1222, 1248, 1196), phospho-HER2/phospho-EGFR (1248/1173), phospho-HER3 (1289), phospho-EGFR (1068, 1046/1047, 1045), phospho-ACC (79), phospho-AMPK substrates (LXRXpS/pT), AMPK α , AMPK β 1/2, ACC, PARP, HER2, EGFR, HER3, LKB1, FASN and Myc-tag primary antibodies were purchased from Cell Signaling Technology. Anti-rabbit and anti-mouse secondary antibodies were also purchased from Cell Signaling Technology. Anti-phospho-EGFR (1142) was purchased from ECM Biosciences. Anti-Actin antibody was purchased from Sigma-Aldrich. 5-Amino-4-imidazole carboxamide riboside (AICAR), a pharmacological activator of AMPK, was purchased from Toronto Research Chemicals (Toronto, ON, Canada). AICAR was dissolved in dimethyl sulfoxide (DMSO) to make a 500mM stock concentration. Morin hydrate was purchased from Sigma-Aldrich and dissolved in DMSO to make a 50mM stock solution. C75 was purchased from Sigma-Aldrich. 2-Deoxy-D-glucose (Sigma-Aldrich) was dissolved in Phosphate Buffered Saline (PBS) to a stock solution of 2M. 2,5-dideoxyadenosine (Sigma-Aldrich) and lapatinib ditosylate (LC Laboratories) were dissolved in DMSO. Metformin hydrochloride (Sigma-Aldrich) was dissolved in PBS to make a 500mM stock solution. Epidermal growth factor (EGF) (Sigma-Aldrich) was prepared in a stock concentration of 100 μ g/ml in purified water. Compound C in solution was obtained from Calbiochem. G418 was obtained from Invitrogen. 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA), Hank's balanced salt solution (HBSS) and PBS were purchased from Gibco. Control, AMPK α 1/2, AMPK β 1, and AMPK β 2 siRNAs were

purchased from Santa Cruz Biotechnology. All siRNA transfections were conducted in triplicate over a period of 72 hours using RNAiMax (Invitrogen) according to the manufacturer's recommendation.

Cell Culture

Human cancer cell lines HCC1937, DU4475, BT549, HCC1806, HCC1954, MDA231, HCC1419, H157, H1975, HCC827, SKMES1, U1752, Calu6 were cultured in RPMI media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). BT474 was grown in DMEM/F12 media (Gibco) supplemented with 10% FBS. SUM102 and SUM159 cell lines were grown in Ham's F12 media (Gibco) with 5% FBS supplemented with 5µg/ml insulin (Gibco) and 1µg/ml hydrocortisone (Sigma). MCF7 and Hs578t cell lines were cultured in DMEM media (Gibco) with 10% FBS. SKBR3 was grown in McCoy's media (Gibco) with 10% FBS. MDA468 and MDAMB175VII were grown in L15 media (Gibco) with 10% FBS in 100% atmosphere. MCF10A was grown in DMEM/F12 media supplemented with 5% horse serum (Gibco), 0.5µg/ml hydrocortisone, 10µg/ml insulin, 20ng/ml EGF, 0.1µg/ml cholera toxin (Sigma) and 100µg/ml each of penicillin and streptomycin (Gibco). All cell lines were cultured at 37°C with 5% CO₂. The MCF10A EGFR #7 and MCF10A EGFR #9 cell lines were a kind gift from Dr. Ben Ho Park (Johns Hopkins).

MTT Assay

MTT assay was performed with the CellTiter 96® Non-Radioactive Cell Proliferation assay (Promega) according to the manufacturer's instruction. Treatments were done in triplicate.

cAMP Measurements

The cAMP Complete ELISA Kit (Enzo Life Sciences) was utilized to measure cAMP levels in breast cancer cells according to the manufacturer's instructions.

Cell Cycle Analysis

SKBR3 and MCF7 cell lines were grown to confluency in 100mm cell culture plates, then treated with DMSO or 1mM AICAR for a period of 24 or 48 hours. At the designated timepoint, cells were trypsinized with 0.25% Trypsin-EDTA and fixed with 1:1 methanol:acetone. Fixed cells were stained with propidium iodide (Calbiochem) for 1 hour. PI fluorescence of the samples was determined by FACSCalibur in the FL-3 channel.

Cell Viability Assay

Cells were trypsinized and plated at a density of 2000 cells per well of a 6-well plate. Plates were treated, in triplicate, 24 hours later with increasing concentrations of AICAR, EGF or both. Cells were cultured 8-21 days under treatment, and then fixed with 100% ethanol. After ethanol fixation, plates were stained with 0.5% crystal violet (Sigma). Cell viability was quantified by dissolving the crystal violet with 1% SDS and measuring absorbance with xMark™ Microplate Absorbance Spectrophotometer (BioRad) at 570nm.

Western Blot Analysis

For measurement of protein levels, cell lysate was collected in TNE lysis buffer. Protein concentration was determined by the Pierce BCA assay (Thermo Fisher Scientific). Protein from each sample was separated by SDS-PAGE on a 10% Tris-HCl gel (Bio-Rad). Protein was then transferred to a nitrocellulose membrane (Bio-Rad) and incubated overnight with the primary antibody. Most antibodies were incubated at a

1:1000 concentration, with the exception of anti-actin antibody (1:10,000). After 1 hour incubation with the anti-rabbit or anti-mouse secondary antibody, membranes were developed with SuperSignal West Femto Max Sensitivity Substrate (Thermo Fisher Scientific). For further analysis, membranes were stripped with Stripping Buffer (Thermo Fisher Scientific) and reprobed with subsequent primary antibodies.

Constitutively Active and Dominant Negative AMPK α

AMPK α Constitutively Active (CA) and Dominant Negative (DN) myc-tag labeled constructs in pcDNA3 (Invitrogen) plasmids were generously provided by Dr. David Carling (MRC Clinical Sciences Centre, Imperial College, London, UK). E. Coli DH5 α was made competent via CaCl₂ treatment then transformed with control (empty pcDNA3 vector), AMPK α CA or AMPK α DN plasmid. Transformed bacteria were grown under selection on ampicillin containing LB-agar plates. DNA was isolated from bacterial colonies using the PureLink® HiPure Plasmid Midiprep kit (Invitrogen). HCC1419 and SKBR3 cell lines were transfected in a 6-well plate with plasmid DNA using Lipofectamine® 2000 (Invitrogen). After 2 days, transfected cells were grown under 150 μ g/ml G418 selection (Invitrogen). Cells were grown to confluency and cell lysate obtained. Western blot analysis was conducted to confirm the presence of AMPK α CA /AMPK α DN using myc-tag antibody (Cell Signaling Technology).

Immunoprecipitation

Cells were harvested in lysis buffer (50mM Tris, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) and supernatants incubated with rotation at 4oC with either anti-HER2 or anti-EGFR antibody overnight. Protein G-Agarose (Roche Diagnostics) was added the next day, and lysates were placed on the rotator at 4oC for 4 hours. Protein G-

agarose beads were isolated by centrifugation, washed three times with lysis buffer and heated for 5 minutes at 100°C in loading buffer. Samples were run on SDS-PAGE and then probed by immunoblot for HER2, AMPK α , and an antibody specific for phosphorylation of AMPK consensus site (anti-phospho-AMPK substrates).

***In vitro* Kinase Assay**

AMPK ($\alpha 1\beta 1\gamma 1$) was purchased from SignalChem and assay conducted via manufacturer's protocol with Kinase Dilution Buffer VII (SignalChem) and Kinase Assay Buffer I (SignalChem). [^{32}P]-ATP was obtained from PerkinElmer. ATP and AMP were purchased from Sigma. *In vitro* AMPK inhibition was obtained with the addition of Compound C to the assay. Peptides were synthesized by the Johns Hopkins Synthesis and Sequencing Facility. Peptides synthesized were as follows: HA Peptide (NH₂-YPYDVPDYA-OH), HER2#1 (NH₂-TLERPKTLSPGRR-OH), HER2#2 (NH₂-SILRRRFTHQSDVRR-OH), HER2#2A (NH₂-SILRRRFAHQSDVRR-OH), EGFR#1 (NH₂-FLQRYSSDPTGRR-OH), EGFR#2 (NH₂-SILHRIYTHQSDVRR-OH), EGFR#2A (NH₂-SILHRIYAHQSDVRR-OH). Peptides were tested in triplicate. Samples were blotted on P81 Whatman® Cellulose Paper (Sigma). Radioactivity was counted for the samples in the presence of scintillation fluid in a scintillation counter.

Statistical Analysis

Quantitative data were graphed and analyzed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Error bars represent standard error unless mentioned. Student's unpaired t tests were used for analysis of statistical differences. Differences were considered significant at $p < 0.05$.

Chapter 3: AMPK Regulates HER2 and EGFR Activation

3.1 Introduction

As the critical sensor of cellular energy, AMP-activated Protein Kinase (AMPK) is considered the master metabolic regulator of the eukaryotic cell [68]. AMPK is activated in conditions that increase the cellular ADP:ATP or AMP:ATP ratio [82, 144, 145]. Activation of AMPK has been reported to occur in response to exercise, ischemia, glucose deprivation, and genotoxic and oxidative stresses [144]. AMPK functions as a heterotrimer, composed of alpha, beta and gamma subunits [75]. AMP binds the gamma subunit of AMPK directly, which favors LKB1-mediated phosphorylation of AMPK α residue threonine 172 [144]. LKB1, the major upstream kinase of AMPK, is a known tumor suppressor [178]. As a serine-threonine kinase, AMPK is involved in a variety of cellular pathways [66, 75, 84, 87, 146, 147, 149, 152, 157, 158, 161, 163, 168, 169, 175, 177]. Downstream targets of AMPK include: acetyl CoA carboxylase (ACC), raptor, HMG-CoA reductase, unc51-like autophagy activating kinase 1 (ULK1) and tuberlin [67, 145, 161]. Primarily, AMPK maintains energy homeostasis by turning on catabolic pathways that generate ATP, while switching off anabolic pathways that consume ATP [75]. AMPK is also reported to regulate mitochondrial biogenesis, autophagy, cell growth and proliferation, and regulate cell polarity [71, 160, 173].

Several studies have focused on AMPK as a potential target for cancer therapy. As AMPK couples energy status to growth, AMPK functions as a metabolic checkpoint [70]. Several oncogenic mutations and signaling pathways functionally bypass the AMPK-dependent metabolic checkpoint [71, 73, 182]. A study investigating AMPK activation levels in primary breast cancer determined AMPK activation was down-

regulated in 90% of breast cancer patients [181]. Furthermore, the AMPK-upstream activating kinase LKB1 is known to be functionally inactive in ~20% non-small cell lung cancers [71]. Moreover, diabetic patients given metformin, an AMPK activating biguanide, were observed to have a lower incidence of breast cancer as compared to diabetic patients without metformin treatment [98, 100-105]. In the lab, metformin treatment has been determined to reduce tumor growth in xenograft, transgenic, and carcinogen-induced mouse cancer models [179, 180]. Furthermore, tumor-prone mice treated with three different AMPK activators (metformin, phenformin or A769662) displayed significantly delayed tumor development [144, 179].

In this section, we describe a novel interaction between AMPK and the HER2 and EGFR tyrosine protein kinases. We observed increased sensitivity of HER2 and EGFR overexpressing breast cancer cell lines to treatment with the AMPK activating agent 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR). AICAR is converted to IMP, an analog of AMP, in the cell and thereby activates AMPK directly [93]. Our results suggest AMPK directly phosphorylates EGFR and HER2, leading to down-regulation of EGFR and HER2 activation and subsequently to tumor cell death. In this section, we describe a novel cell signaling interaction between HER2/EGFR and AMPK, which not only has biological relevance, but holds potential as a novel therapy strategy for the treatment of HER2 and EGFR expressing breast cancers.

3.2 Results

3.2.1 Activation of AMPK Decreases Breast Cancer Cell Viability

To determine what effects AMPK activation might have on proliferation and survival of breast cancer cells, we first screened a series of breast cancer cell lines for

survival after treatment with AICAR, the canonical activator of AMPK. As shown in Figure 1, we observed considerable variability in the sensitivity of breast cancer cell lines to AICAR treatment with a remarkable correlation between HER2-positive status and high sensitivity to AMPK activation. EGFR-expressing triple negative breast cancer cell lines appeared similarly sensitive, but breast cancer cell lines with low levels of both of these oncogenes showed relatively low sensitivity to AICAR. To confirm the results of our initial MTT assay screen for sensitivity, we conducted colony forming assays of breast cancer cell lines under continuous exposure to AICAR. As shown in Figures 2-4, breast cancer cell lines with high expression of HER2 and/or EGFR were again much more sensitive to AICAR treatment.

To confirm the relationship between high HER2 or EGFR levels and sensitivity to AICAR, we tested MCF10A cells that were engineered by retroviral transduction to stably overexpress EGFR. Two independently EGFR overexpressing clones (MCF10A EGFR#7 and MCF10A EGFR #9) were compared to the parental MCF10A cell line using a colony forming assay to measure response to AICAR. As shown in Figure 5, MCF10A EGFR#7 cells and MCF10A EGFR#9 cells displayed increased sensitivity to AICAR treatment as compared to the parental MCF10A cell line, suggesting that EGFR overexpression increases sensitivity to AMPK-activating agents.

In contrast to cells with high levels of HER2 or EGFR, MCF7, an ER-positive breast cancer cell line with negligible HER2 and EGFR expression, was relatively resistant to AICAR treatment (Figure 3). Comparing responses of MCF7 and SKBR3 to AICAR treatment by flow cytometry cell cycle analysis confirmed the greater sensitivity of the HER2-positive SKBR3 cells, with 49% of cells represented as sub-G₁ fragments

after 24 hour treatment with 1mM AICAR (Figure 6). Noting the fragmentation of SKBR3 cells after treatment with AICAR, we sought to define apoptosis as a mechanism of cell death in these cells. We observed increased levels of cleaved PARP in SKBR3 under AICAR treatment, which is consistent with apoptosis being the mechanism of cell death (Figure 8).

3.2.2 AMPK activation reduces the level of HER2 and EGFR autophosphorylation

Given that high expression of HER2 and EGFR in breast cancer cell lines correlates to increased sensitivity to AMPK activation, we sought to determine the connection between AMPK activation and the EGFR/HER2 pathway. To test whether AICAR's anti-growth effect is mediated by limiting HER2 and EGFR signaling, we assessed the effect of AMPK activation on the main activating autophosphorylation sites of HER2 and EGFR. As shown in Figures 13 and 14, AMPK activation results in diminished HER2 and EGFR activating phosphorylation. Furthermore, extended treatment of AICAR on EGFR-overexpressing cell lines results in decreased levels of total EGFR protein (Figures 15 and 16).

3.2.3 Genetic studies confirm the role of AMPK in the regulation of HER2 and EGFR activation

To confirm that the inhibitory effect of AICAR on HER2 and EGFR phosphorylation is mediated by AMPK, we transfected SKBR3 cells with AMPK siRNA. As a heterotrimer, functional AMPK is composed of α , β , and γ subunits; we found that AMPK β siRNA was more efficacious than AMPK α in the knockdown of AMPK protein (Figure 17). Under conditions of AMPK knockdown (using AMPK β siRNA), we observed increased activating phosphorylation of EGFR and HER2 (Figures 17 and 18).

In additional experiments, transfection of HCC1419 and SKBR3 cells with AMPK α constitutively active (CA) and dominant negative (DN) Myc-tag labeled constructs further validated the regulatory role of AMPK on HER2 and EGFR. Myc-tag expressing clones were isolated under G418 antibiotic selection and analyzed for HER2 and EGFR phosphorylation status (Figures 19-21). Similar to the effect of AICAR treatment, AMPK CA clones demonstrated decreased phosphorylation of HER2 and EGFR (Figures 19-21). Taken together, these results strongly support the notion of AMPK mediating an inhibitory effect on HER2 and EGFR activation.

3.2.4 EGFR ligands rescue breast cancer cells from AMPK-based cytotoxicity

To determine whether AMPK-mediated inhibition of HER2 and EGFR is responsible for the cytotoxic effects in HER2/EGFR overexpressing cell lines, we assessed whether stimulation of the EGFR/HER2 pathway would have a rescue effect. As shown in Figures 22 and 23, concurrent treatment of HER2-positive breast cancer cells with AICAR and EGF resulted in a rescue effect. When compared to AICAR treatment alone, concurrent AICAR and EGF treatment result in a 31% rescue of colony forming efficiency in HCC1419 (Figure 22). These results suggest the cytotoxic effects of AMPK activation are mediated mainly through inhibition of HER2/EGFR signaling. By stimulating HER2/EGFR signaling through EGF treatment, AMPK-mediated inhibition of HER2/EGFR is counterbalanced, resulting in a rescue of cell viability.

3.2.5 AMPK directly binds HER2 and phosphorylates HER2 and EGFR

Given that our results suggest AMPK-dependent regulation of HER2/EGFR activation, we next set out to determine whether AMPK regulation of HER2/EGFR is a result of a direct interaction (Figure 27) of AMPK with these receptor tyrosine kinase

proteins. Our analysis of HER2 and EGFR amino acid sequences revealed that each protein has two sequences that potentially fit the AMPK substrate consensus motif (Figure 24), which is LXRXX(S/T), where X represents any amino acid. Using an antibody that recognizes the phosphorylated LXRXX(S/T) motif (Phospho-(Serine/Threonine) AMPK Substrate), we immunoprecipitated HER2 and EGFR and then probed the precipitated proteins with the phospho-(S/T) AMPK substrate sequence. These experiments revealed that both EGFR and HER2 harbor the AMPK substrate motif (Figure 25). Furthermore, the presence of the phosphorylated LXRXX(S/T) motif increased with treatment of AICAR, indicating that AMPK directly phosphorylates HER2/EGFR (Figure 25). We also performed co-immunoprecipitation experiments to determine if AMPK directly interacts with HER2/EGFR, and as shown in Figure 26, AMPK co-immunoprecipitates with HER2. These results confirm that AMPK negatively affects EGFR and HER2 activation through a direct interaction.

3.2.6 AMPK *in vitro* kinase assay reveals phosphorylation sites on HER2 and EGFR for AMPK

To determine which of the two possible phosphorylation sequences on HER2 and EGFR represented the site of AMPK phosphorylation, we designed an *in vitro* kinase assay experiment. We synthesized four peptides corresponding to the potential phosphorylation sites, which we designate as HER2a, HER2b, EGFRa, and EGFRb (Figure 28). Based on the results of the *in vitro* assay, we determined that HER2b and EGFRb correspond to the specific sites of AMPK phosphorylation (Figure 29). To validate these sites of AMPK phosphorylation, we synthesized two additional peptides based on the sequence of HER2b and EGFRb, wherein the prospective phosphorylated

threonine was changed to an alanine: HER2b^{T→A} and EGFRb^{T→A} (Figure 28). The inability of AMPK to phosphorylate these altered peptides confirmed that HER2 threonine 900 and EGFR threonine 892 are specific phosphorylation sites for AMPK. These results also provide additional confirmation that AMPK inhibits HER2 and EGFR activity through a direct phosphorylation event. Moreover, our discovery of AMPK-mediated regulation of HER2/EGFR signaling provides an explanation for the heightened sensitivity observed in HER2/EGFR overexpressing breast cancer cell lines to the AMPK activating agent, AICAR.

3.3 Discussion

In summary, this series of experiments demonstrate that activated AMPK preferentially inhibits the growth of HER2/EGFR-dependent breast cancer cells. AMPK has several downstream targets and is a key player in regulating cell growth and metabolism processes. We show that AMPK activation results in cell growth inhibition through the disruption of HER2/EGFR signaling. AMPK activation negatively regulates HER2/EGFR signaling, which has a direct, negative impact on breast cancer cell survival. Our results suggest that AMPK activating agents may have therapeutic benefit for HER2/EGFR-dependent breast cancers.

EGFR and HER2, of the ErBb family of tyrosine protein kinases, are implicated in driving several important types of human cancers. EGFR is overexpressed in some breast, bladder, kidney, non-small lung, and prostate cancers [42, 49, 50], whereas HER2 is overexpressed in breast, colon, endometrial, lung, cervix, esophageal and pancreatic cancers [184]. To mediate cell growth and proliferation, EGFR and HER2 must be activated through a multi-step process that involves ligand binding, homo- or

heterodimerization, and autophosphorylation of key tyrosine residues in the intracellular domain [185]. When activated, both HER2 and EGFR drive cell growth through downstream signaling pathways [184]. MAPK and Akt pathways, two such downstream pathways, are especially well-studied for their role in cancer biology [186]. The importance of EGFR and HER2 signaling is evidenced by the surfeit of overexpression or activating mutations of these proteins in cancer [184, 187]. HER2 is overexpressed in ~25% of breast cancer and as many as 15% of breast cancers overexpress EGFR [184, 188]. Furthermore, co-overexpression of HER2 and EGFR has been linked to poor clinical outcome in breast cancer patients [187].

Being key players in cancer development and growth, HER2 and EGFR have emerged as attractive targets for pharmacological intervention [49, 61]. Currently, both immunological and pharmacological inhibition strategies are practiced in the clinic. Trastuzumab, a humanized monoclonal antibody to HER2, is currently utilized in the treatment of HER2 overexpressing breast cancer and effectively down-regulates surface HER2 [50-53]. Lapatinib, a small molecule dual inhibitor of EGFR and HER2, is also a current therapy option for HER2-positive breast cancer patients [41, 58, 188], although as monotherapy, lapatinib has limited benefit, with a 4.3% response rate in patients with HER2-positive breast cancer [186, 189, 190]. While these therapies have proven efficacious in the treatment of HER2 overexpressing breast cancer, not all HER2-positive cancers respond to these treatments and the development of trastuzumab and lapatinib resistance is a growing issue of concern [191]. Moreover, triple negative breast cancer (TNBC) remains difficult to treat, with a high risk of metastasis and disease recurrence [192]. TNBC is a broad term that encompasses a heterogeneous group of breast cancers

that are defined by their lack of estrogen receptor (ER), progesterone receptor (PR) and HER2 expression [193]. TNBC is usually characterized as aggressive tumors with systemic treatment options limited to cytotoxic chemotherapy [39]. Approximately 30-50% of TNBC overexpress EGFR [194]. The high frequency of EGFR overexpression in TNBC would support the use of EGFR inhibitors as a strategy in TNBC treatment [195]. Although anti-EGFR therapy is expected to be efficacious for TNBC treatment, clinical trials have thus far shown no clinical benefit for a variety of anti-EGFR therapies [195]. Consequently, the development of novel therapies is of vital import for the treatment of TNBC.

Although much is known about EGFR and HER2 activation and subsequent signaling pathways, little is known about the regulation of EGFR and HER2 under conditions of metabolic stress. Conditions of metabolic or environmental stress would not only be a detriment to the cell, but logically would inhibit cell growth signaling pathways. While several proteins are involved in conveying cellular stress response, AMP-activated protein kinase (AMPK) is known as the master metabolic regulator of the cell for its varied functions in regulating lipid, carbohydrate and protein synthesis [67].

In this section, we explored the connection between HER2/EGFR-dependent cell signaling and sensitivity to AMPK activation. Subsequently, we determined a novel regulation-based interaction between AMPK and HER2/EGFR. Specifically, activated AMPK directly binds HER2 and phosphorylates HER2 at threonine 900. Similarly, AMPK recognizes a consensus sequence on EGFR and phosphorylates at threonine 892. Our findings clearly define a novel signaling pathway, whereby AMPK regulates HER2/EGFR activity through direct inhibitory phosphorylation. These results not only

provide a mechanism for efficacy of AMPK agonists in HER2/EGFR-activated breast cancer, but also support the therapeutic use of AMPK agonists in the treatment of HER2 or EGFR-activated breast cancer.

Figure 1

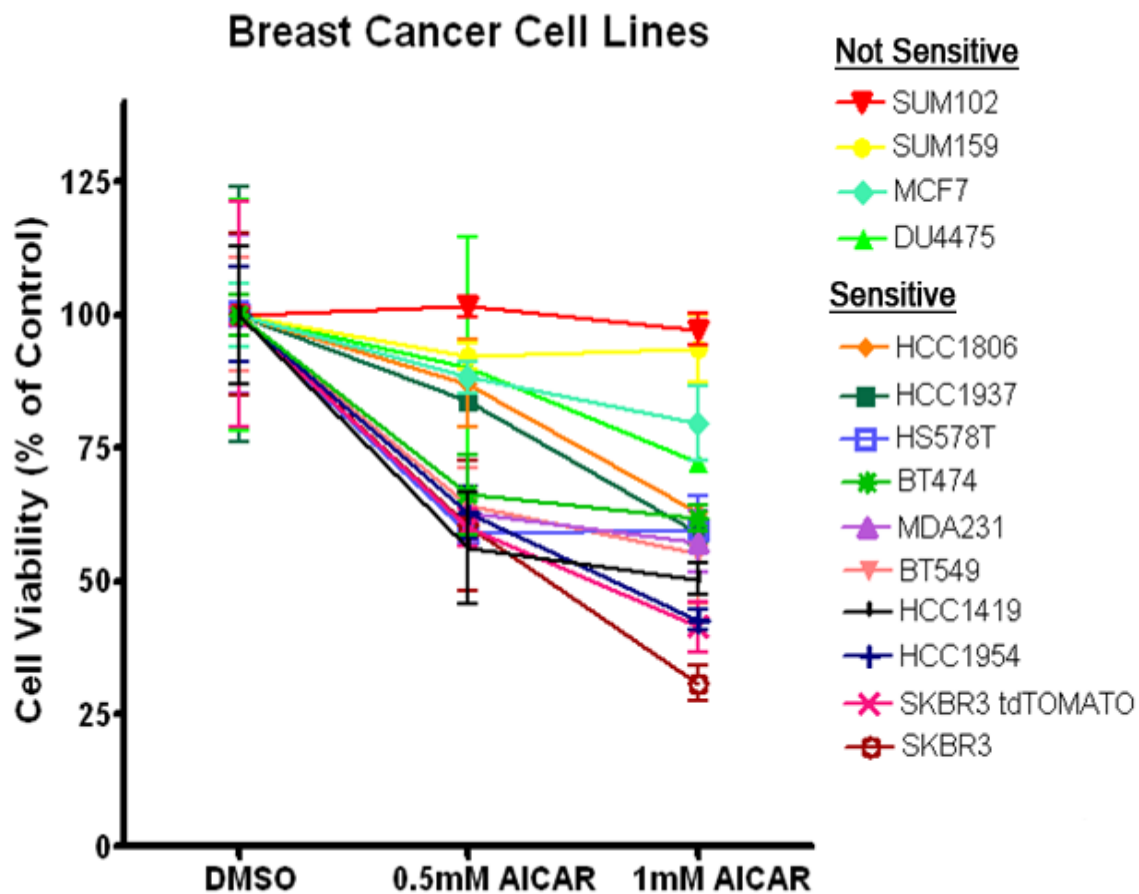


Figure 1: AICAR reduces the survival of HER2-amplified breast cancer cell lines.

MTT assay analysis of breast cancer cell line viability with DMSO control, 0.5mM and 1mM AICAR treatment. Breast cancer cell lines were seeded into a 96-well plate and treated for 24 hours. Treatments were done in triplicates and results were measured using a spectrophotometer at 570nm with a reference wavelength of 690nm. Error bars indicate SEM.

Figure 2

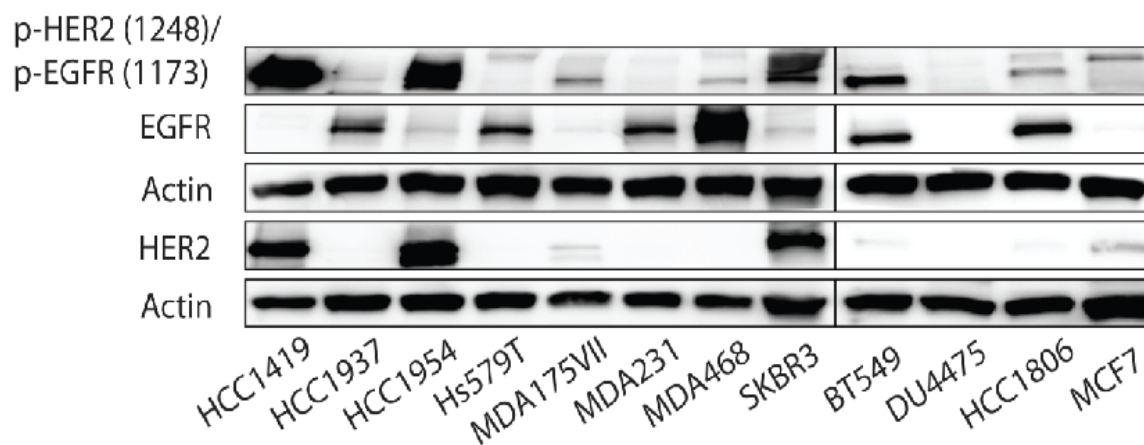


Figure 2: Analysis of HER2 and EGFR levels in selected breast cancer cell lines.

Western blot analysis of HER2 and EGFR levels in selected breast cancer cell lines, showing that the cell lines with greatest sensitivity to AICAR have relatively high levels of HER2, EGFR, or both.

Figure 3

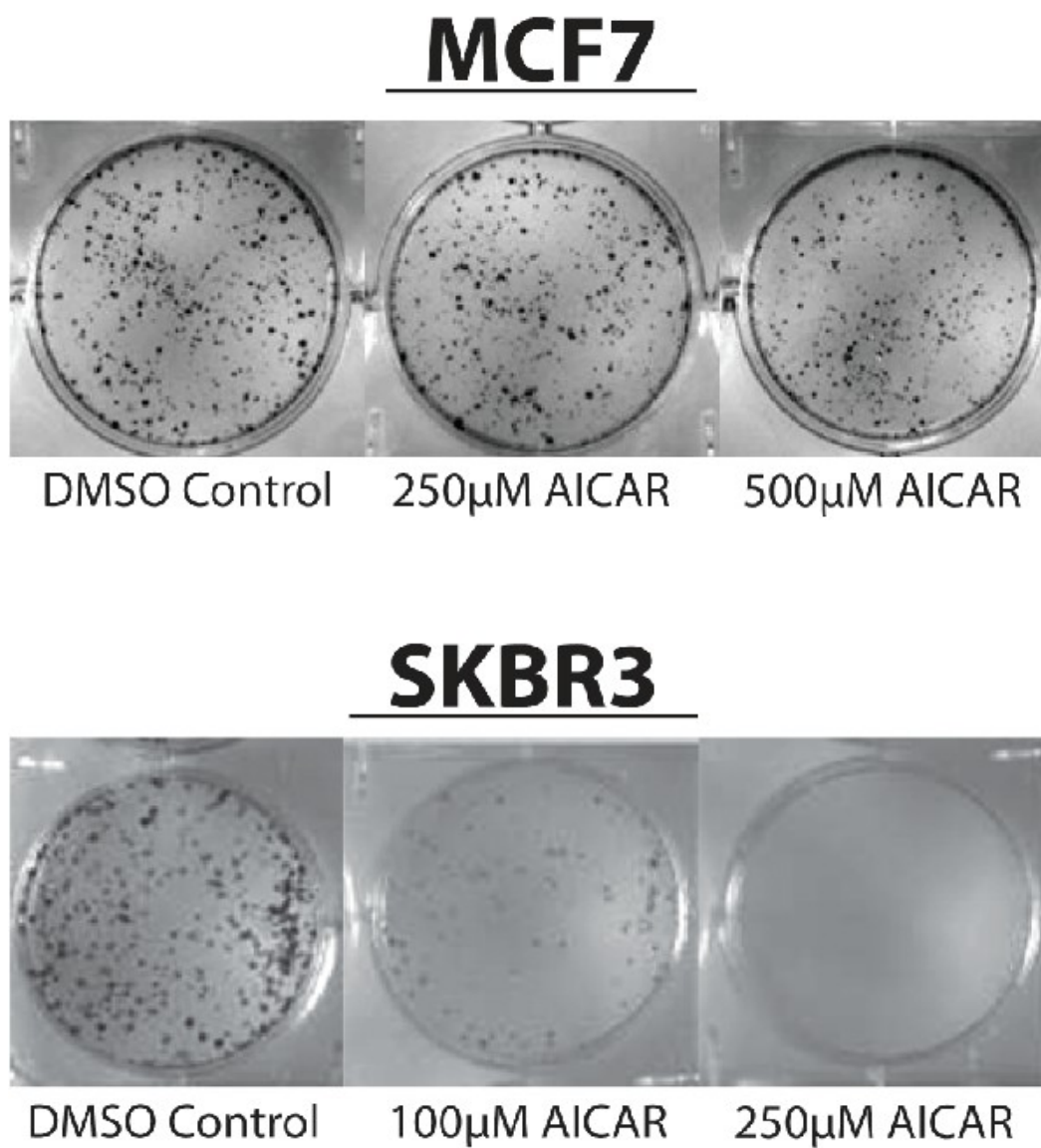
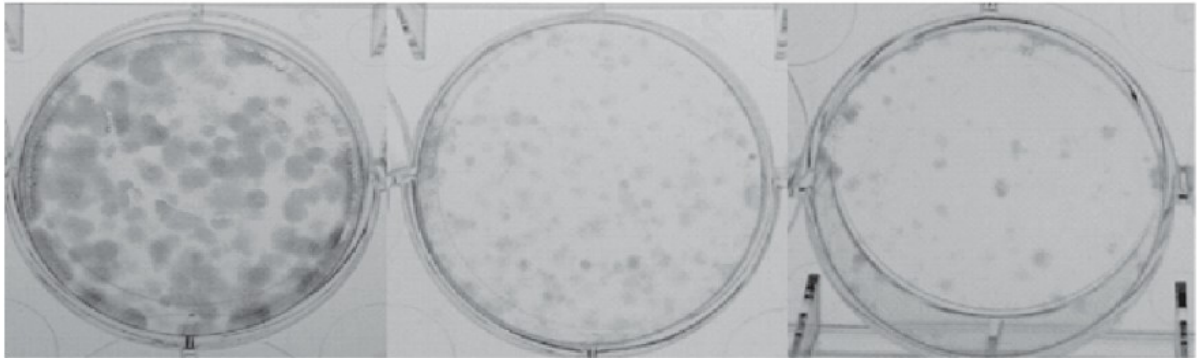


Figure 3: Sensitivity profiles of ER+ and HER2+ breast cancer cell lines to continuous AMPK activation. Clonogenicity (colony forming efficiency) of MCF7 and SKBR3 cells treated with indicated concentrations of AICAR. Colony forming assays were done in triplicate.

Figure 4

MDA231

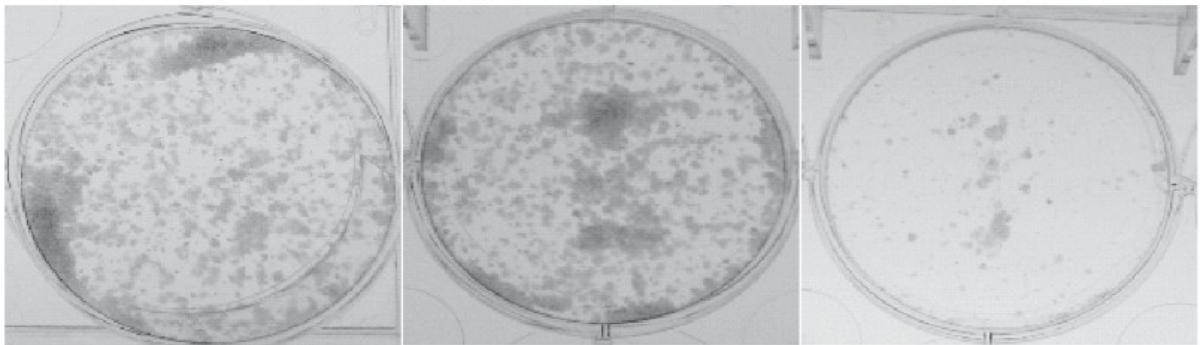


DMSO Control

250µM AICAR

500µM AICAR

HCC1806



DMSO Control

50µM AICAR

100µM AICAR

Figure 4: Cell growth inhibition in triple negative breast cancer cell lines exposed to continuous AMPK activation. Clonogenicity of MDA231 and HCC1806 cells – two cell lines with high levels of EGFR expression – treated with the indicated concentrations of AICAR. Cells were treated for 2 weeks then fixed and stained. Colony forming assays were done in triplicate.

Figure 5

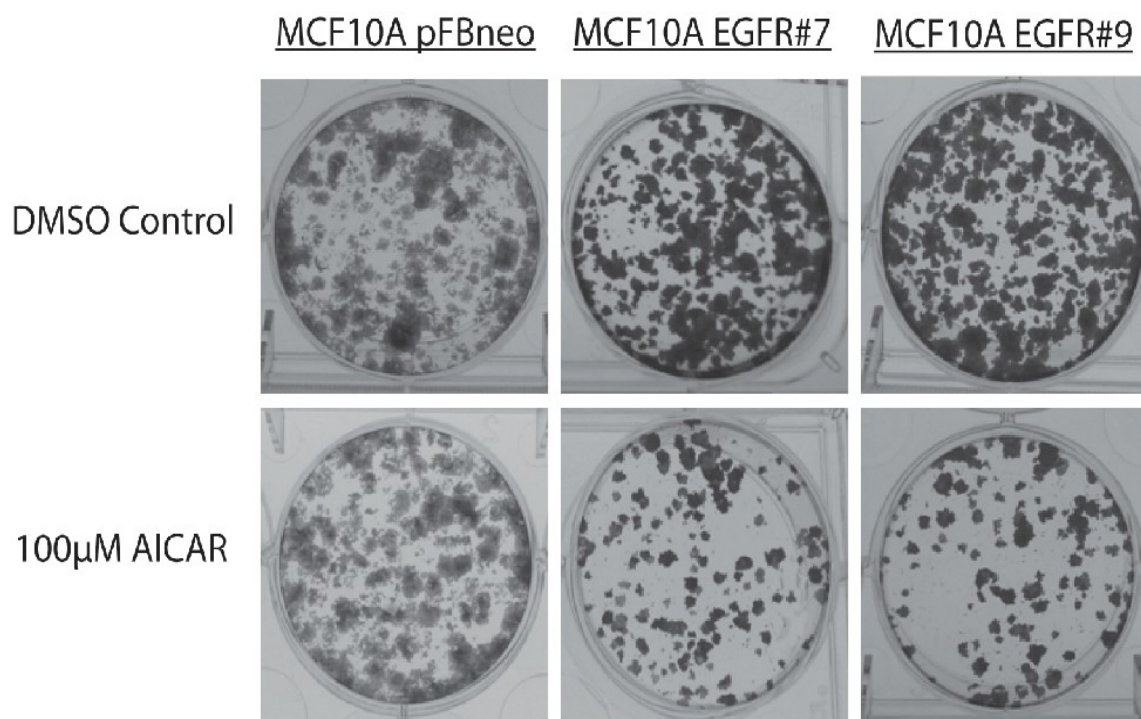


Figure 5: EGFR overexpression in the MCF10A cell line increases sensitivity to AICAR treatment. Clonogenicity of MCF10A empty vector (pFBneo) cells and MCF10A clones with stable overexpression of EGFR (EGFR #7 and EGFR #9) treated with AICAR. Overexpression of EGFR in MCF10A cells results in increased sensitivity to AICAR. Experiments were done in triplicate.

Figure 6

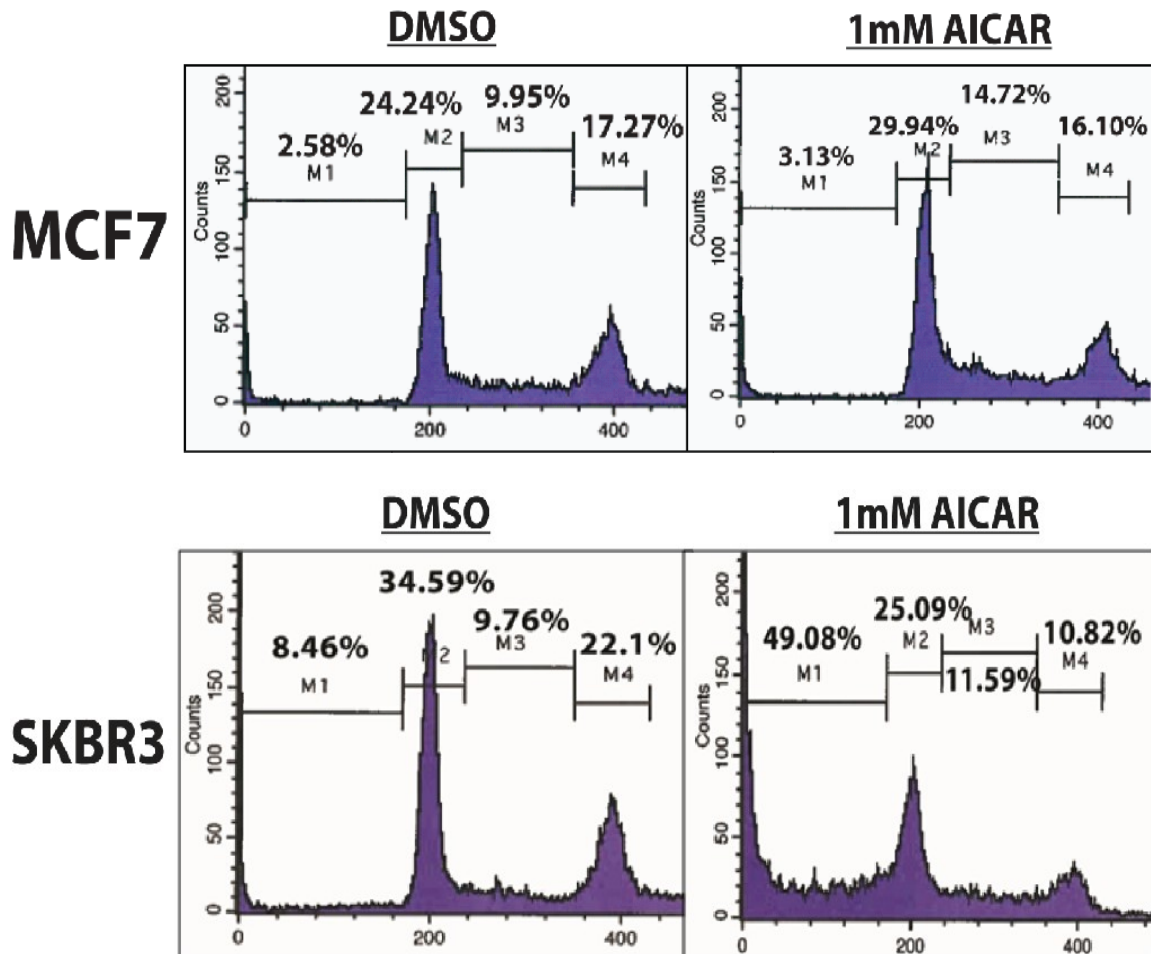


Figure 6: Cell cycle analysis of MCF7 and SKBR3 cells under 24 hour AICAR

treatment. Flow cytometry measurements of cell-cycle distribution of propidium iodide-stained SKBR3 and MCF7 cells treated for 24 hours with either 1mM AICAR or DMSO only. Presence of large sub-G₁ populations in AICAR-treated SKBR3 cells suggests apoptotic death.

Figure 7

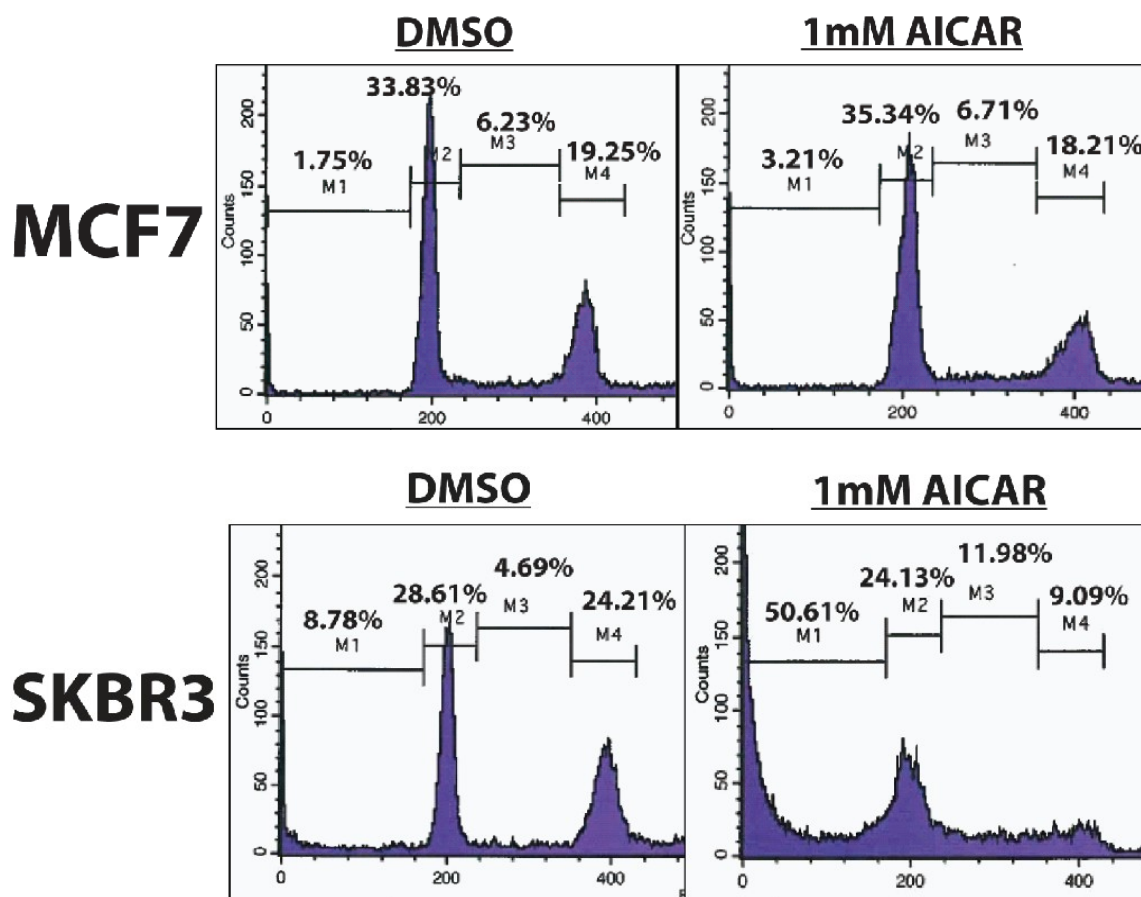


Figure 7: Cell cycle analysis of MCF7 and SKBR3 cells under 48 hour AICAR

treatment. Flow cytometry measurements of cell-cycle distribution of propidium iodide-stained SKBR3 and MCF7 cells treated with either DMSO or 1mM AICAR for 48 hours. Presence of large sub-G₁ populations in AICAR-treated SKBR3 cells suggests apoptosis.

Figure 8

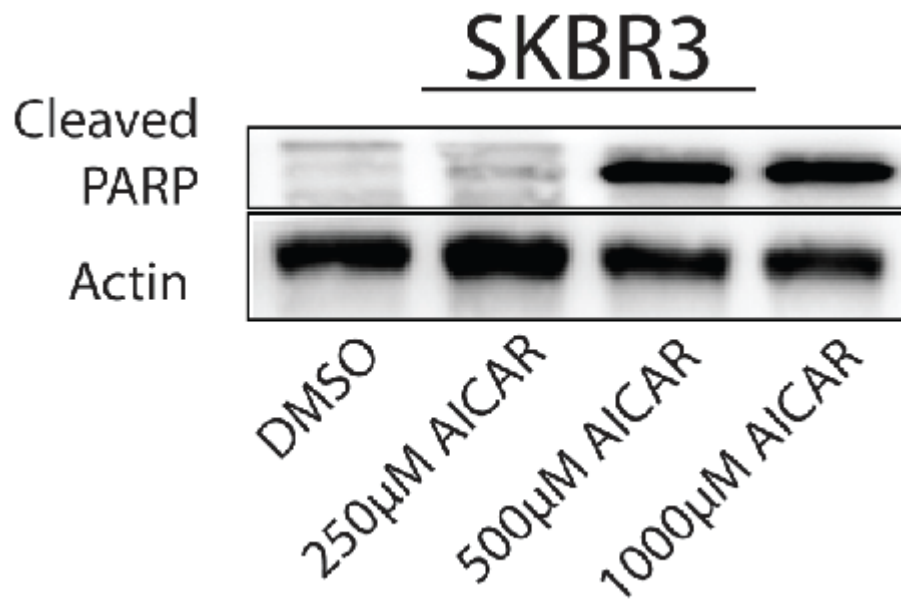


Figure 8: AICAR treatment causes apoptotic cell death in SKBR3 cells. Western blot analysis for cleaved PARP in SKBR3 treated for 24 hours with indicated concentrations of AICAR or DMSO only (control) confirms apoptosis-mediated cell death in SKBR3 cells.

Figure 9

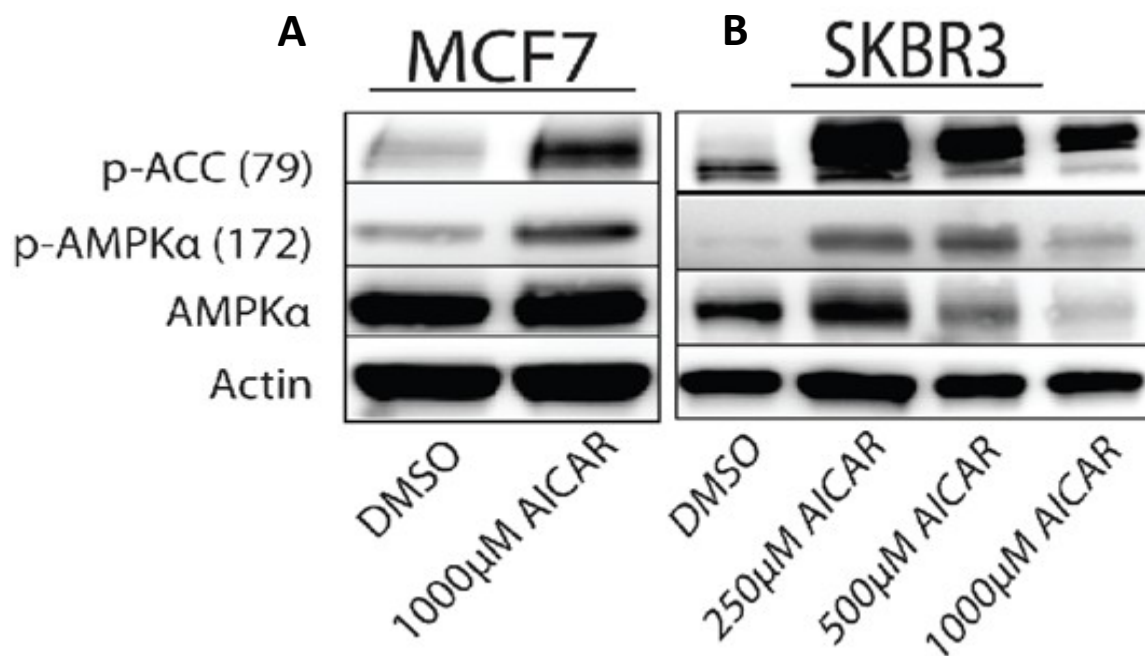


Figure 9: AICAR treatment activates AMPK in breast cancer cell lines. (A) Western blot analysis of MCF7 treated with either 1mM AICAR or (control) for 24 hours. Phosphorylation of AMPK at threonine 172 indicates activation of AMPK. AMPK-mediated phosphorylation of Acetyl Co-A Carboxylase (ACC) at serine 79 confirms AMPK activation. (B) Western blot analysis of SKBR3 treated for 24 hours with DMSO or the indicated concentrations of AICAR.

Figure 10

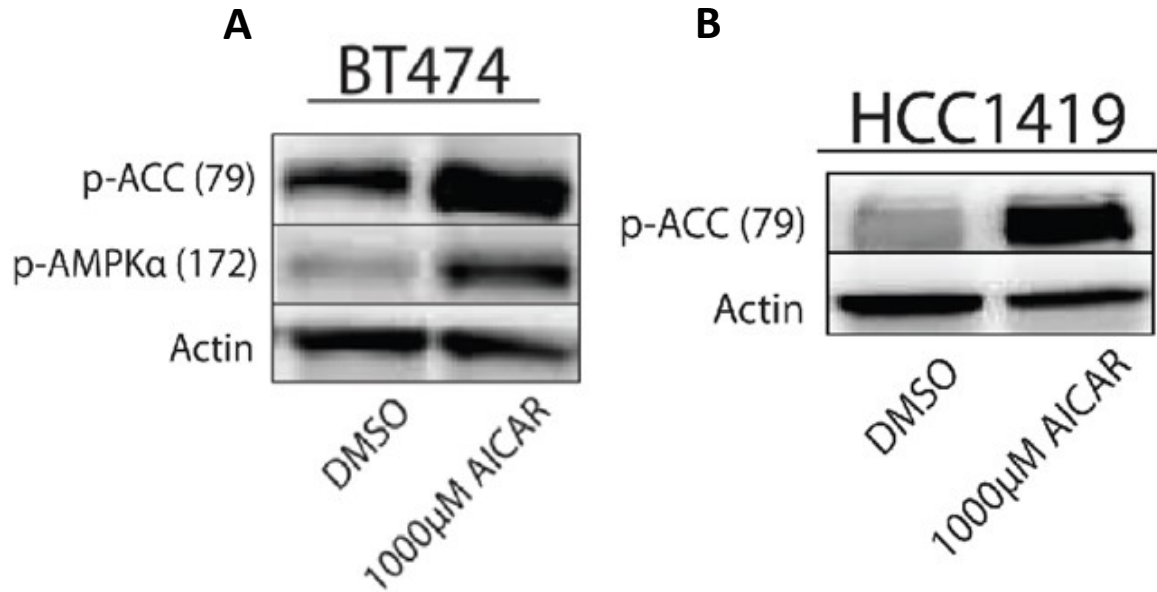


Figure 10: AICAR treatment activates AMPK in HER2+ breast cancer cell lines.

Western blot analysis of 24 hour AICAR treatment in the HER2 positive cell lines BT474 (A) and HCC1419 (B). Phosphorylated ACC indicates activated AMPK.

Figure 11

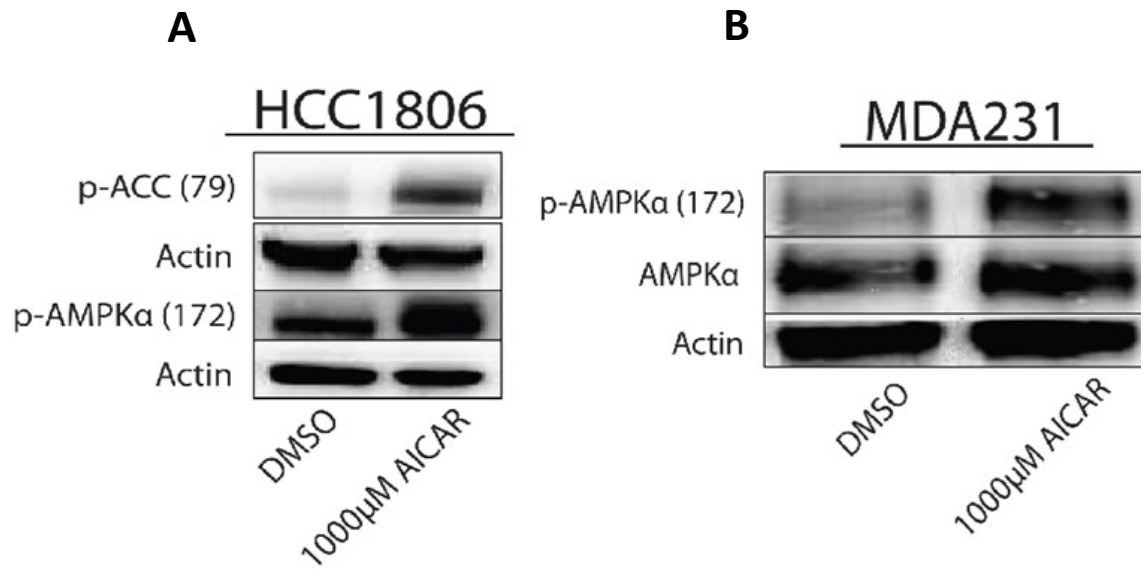


Figure 11: AICAR treatment activates AMPK in triple negative breast cancer cell lines. Western blot analysis of triple negative breast cancer cell lines HCC1806 (A) and MDA231 (B) treated with 1mM AICAR for 24hr (HCC1806) or 72hr (MDA231).

Figure 12

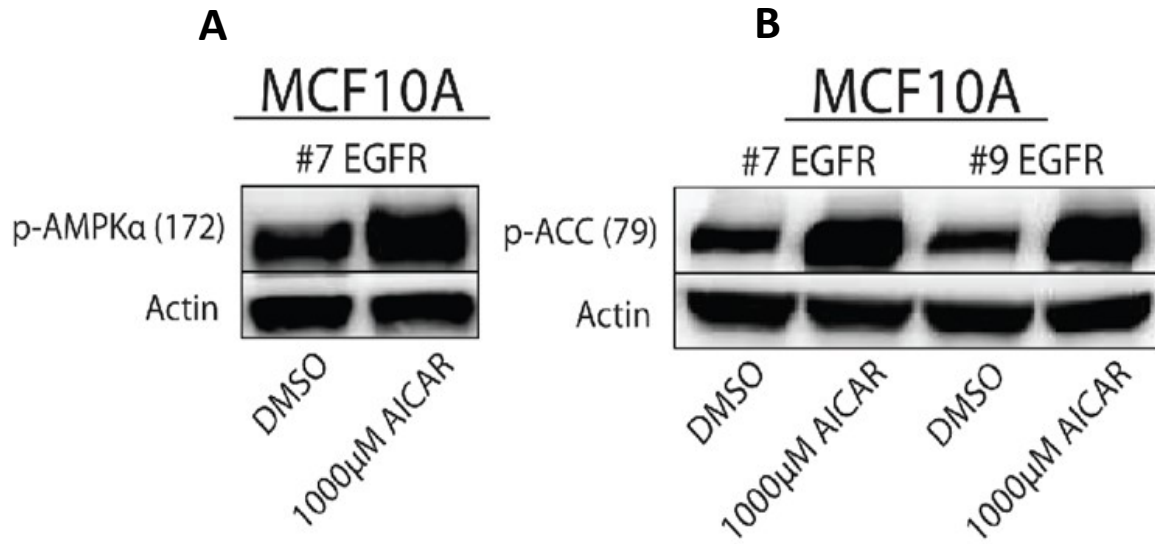


Figure 12: AICAR treatment activates AMPK in the EGFR overexpressing cell lines MCF10A #7 and #9. Western blot analysis of MCF10A and the EGFR overexpressing clones MCF10A #7 EGFR (A and B) and MCF10A #9 EGFR (B) for activation of AMPK following 72hr AICAR treatment.

Figure 13

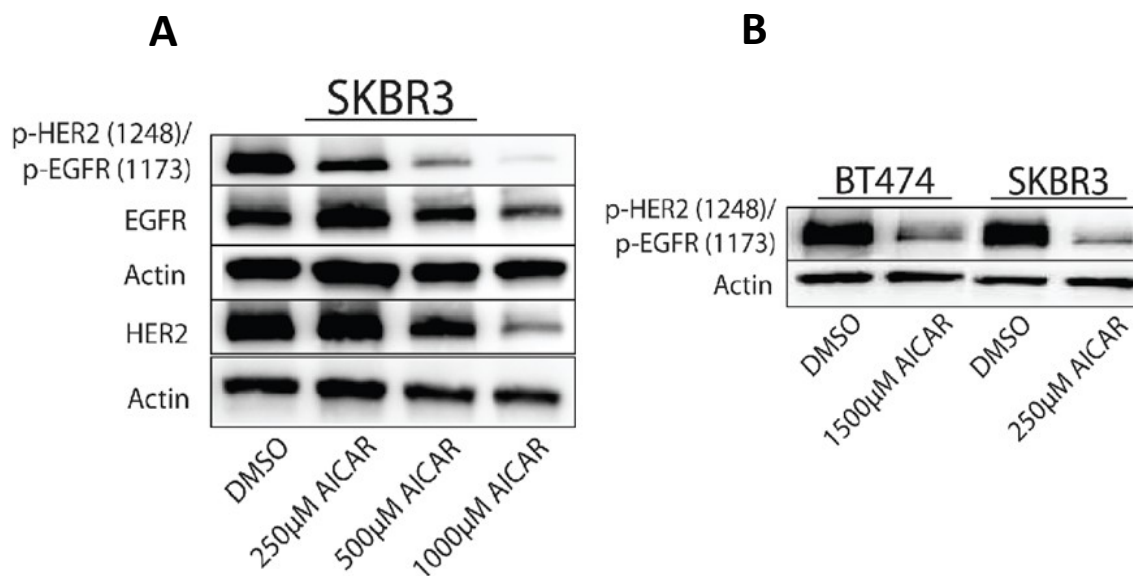


Figure 13: AMPK activation leads to decreased levels of HER2 and EGFR phosphorylation. (A) Western blot analysis shows decreased levels of phosphorylated HER2 and EGFR in SKBR3 cells treated for 24 hours with indicated concentrations of AICAR compared to DMSO control. (B) Similarly, further Western blot analysis of both BT474 and SKBR3 shows decreased phosphorylated HER2 and EGFR after 24hr AICAR treatment.

Figure 14

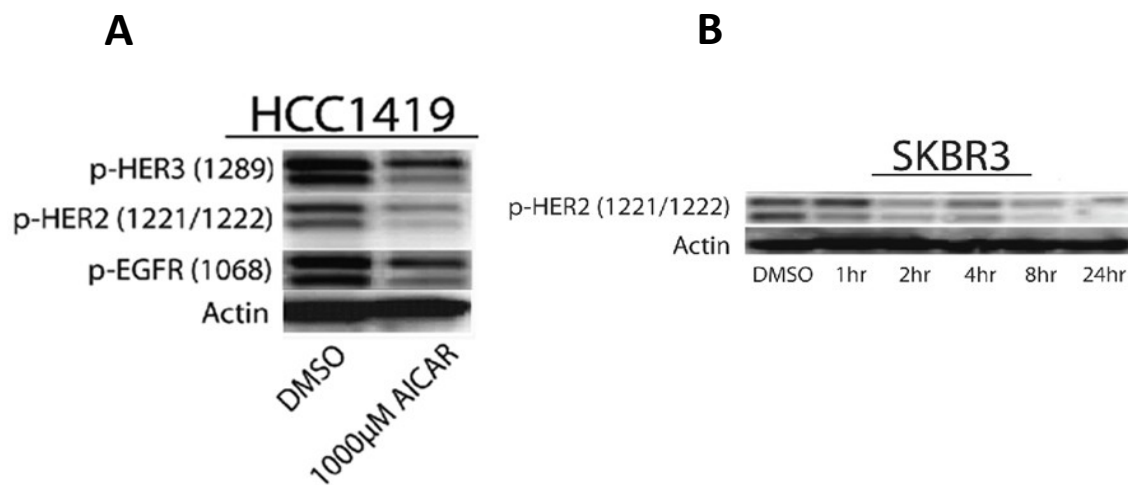


Figure 14: AMPK activation leads to decreased levels of phosphorylated HER2, HER3, and EGFR in HER2+ breast cancer cell lines. (A) Western blot analysis of phosphorylated HER3, HER2, and EGFR protein in HCC1419 treated with either DMSO or 1mM AICAR for 96 hours. (B) Western blot analysis of phosphorylated levels of HER2 in SKBR3 treated with 1mM AICAR for the indicated times.

Figure 15

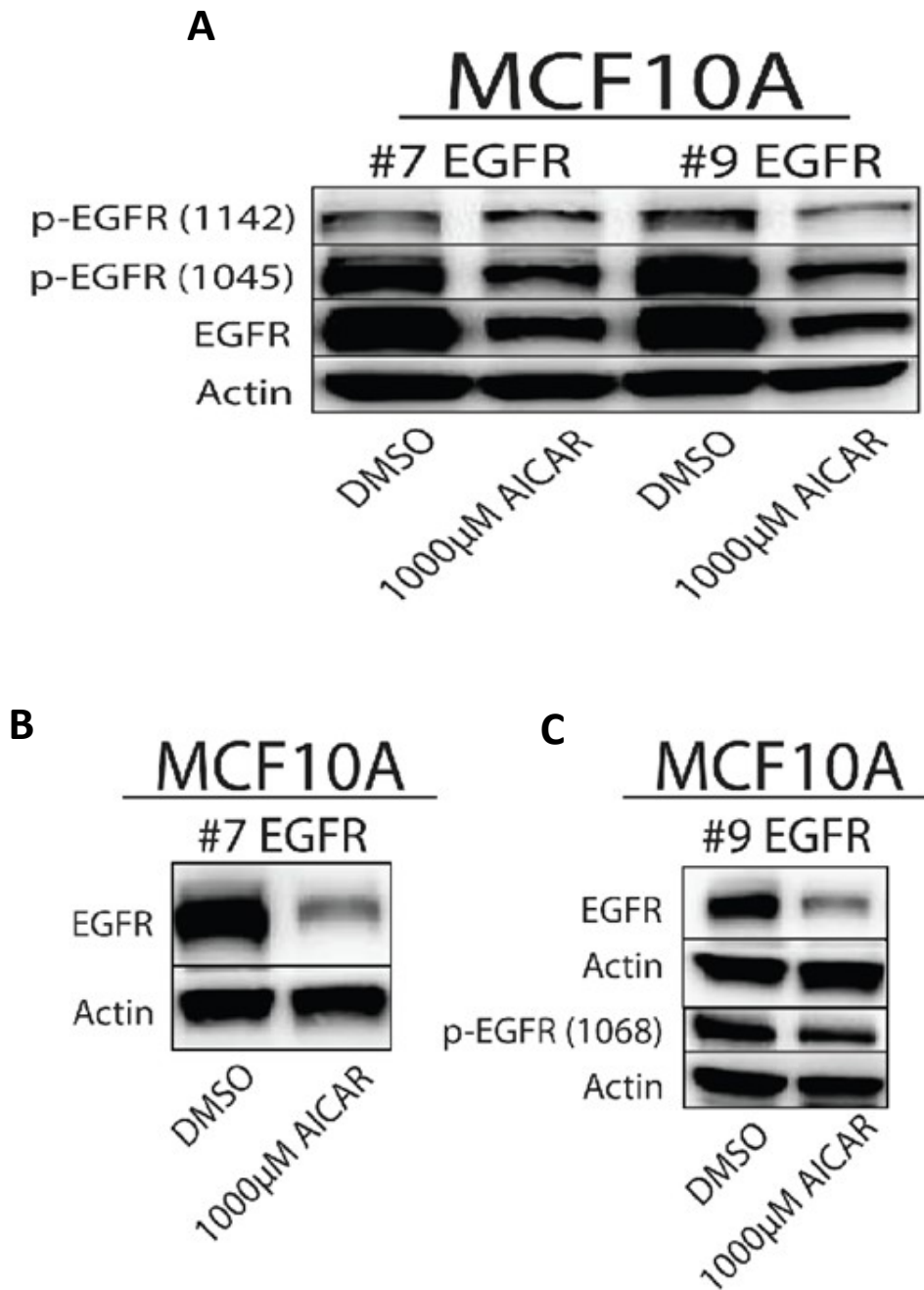


Figure 15: AMPK activation leads to decreased EGFR activation. (A-C) Western blot analysis of the phosphorylated and total level of EGFR protein in MCF10A #7 and #9 EGFR cell lines. Samples were obtained after 72 hour treatment with 1mM AICAR.

Figure 16

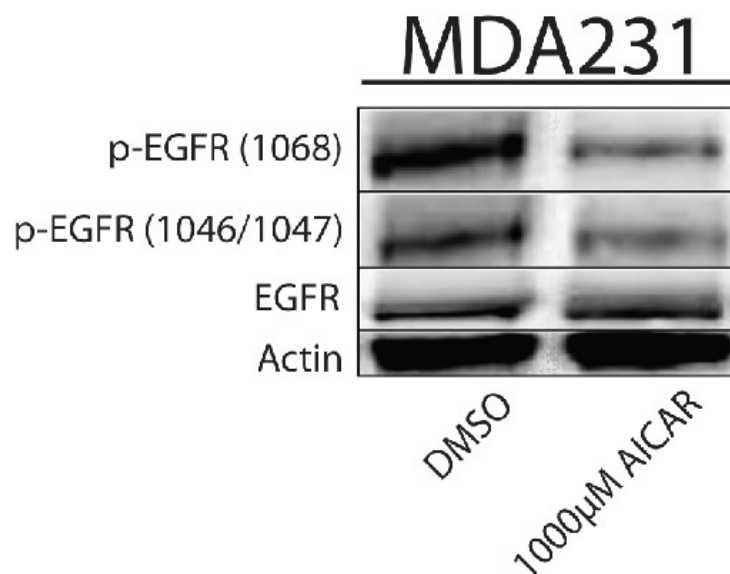


Figure 16: AMPK activation inhibits EGFR activation in triple negative breast cancer. Western blot analysis of phosphorylated and total EGFR levels in MDA231 with 72 hour 1mM AICAR treatment.

Figure 17

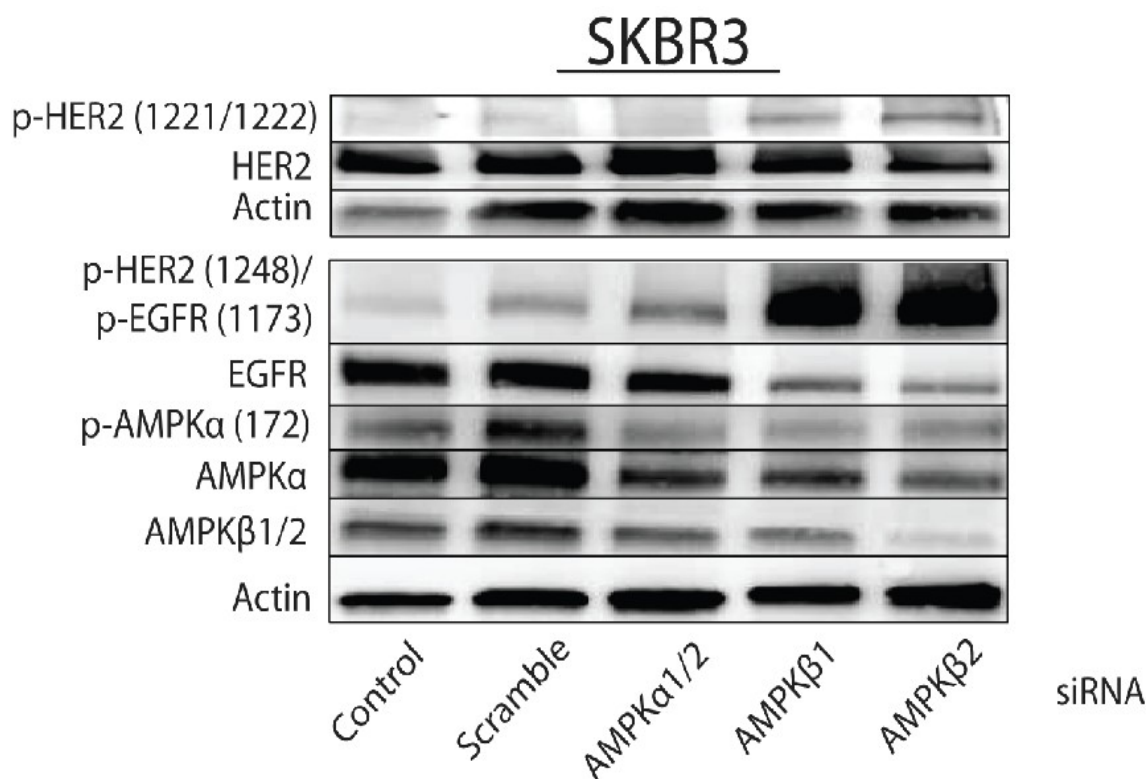


Figure 17: Regulatory role of AMPK in HER2 and EGFR activation is confirmed by genetically regulating AMPK levels and activity. SKBR3 cells were transfected with AMPKα1/2, AMPKβ1, AMPKβ2, or scrambled control siRNA and three days after transfection, cell lysates were evaluated by immunoblot for HER2, p-HER2(1248)/p-EGFR(1173), EGFR, p-AMPKα(172), AMPKα, AMPKβ1/2, and p-HER2(1221-2). Note increased levels of p-HER2 (1248)/p-EGFR (1173) and p-HER2 (1221-2) with AMPKβ1 or AMPKβ2 knockdown, while levels of AMPKα and AMPKβ are decreased by corresponding RNAi.

Figure 18

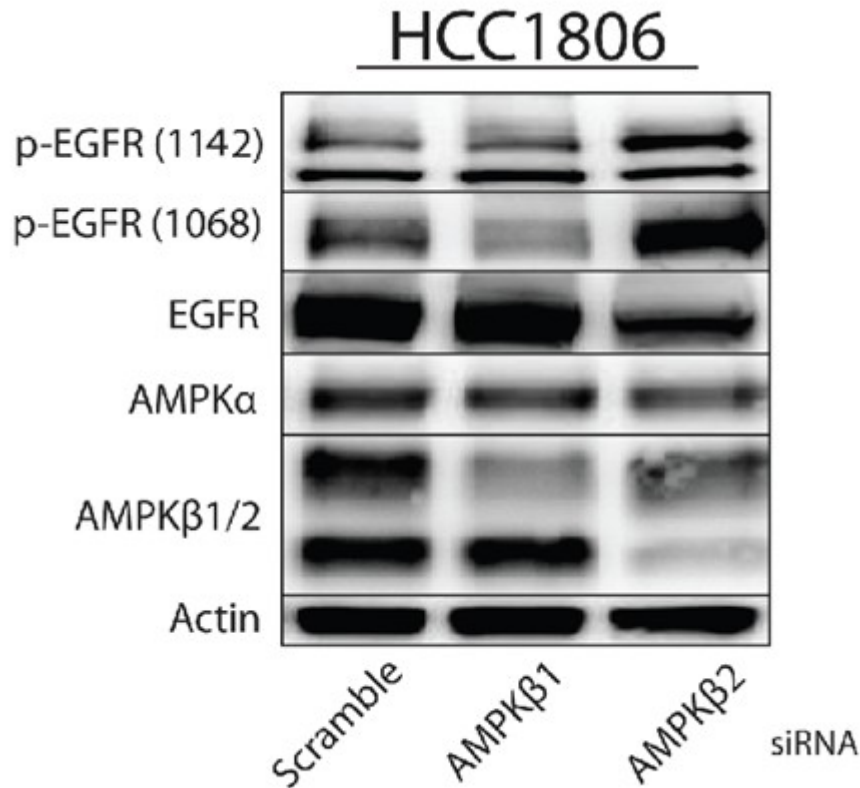


Figure 18: Knockdown of AMPK affects EGFR activation in triple negative breast cancer. HCC1806 cells were transfected with AMPKβ1, AMPKβ2, or scrambled control siRNA and three days after transfection, cell lysates were evaluated by immunoblot for p-EGFR (1142), p-EGFR (1068), EGFR, actin, AMPKα, and AMPKβ1/2. Note increased levels of p-EGFR (1068) and p-EGFR (1142) levels with AMPKβ2 knockdown, while the level of AMPKβ is decreased by corresponding RNAi.

Figure 19

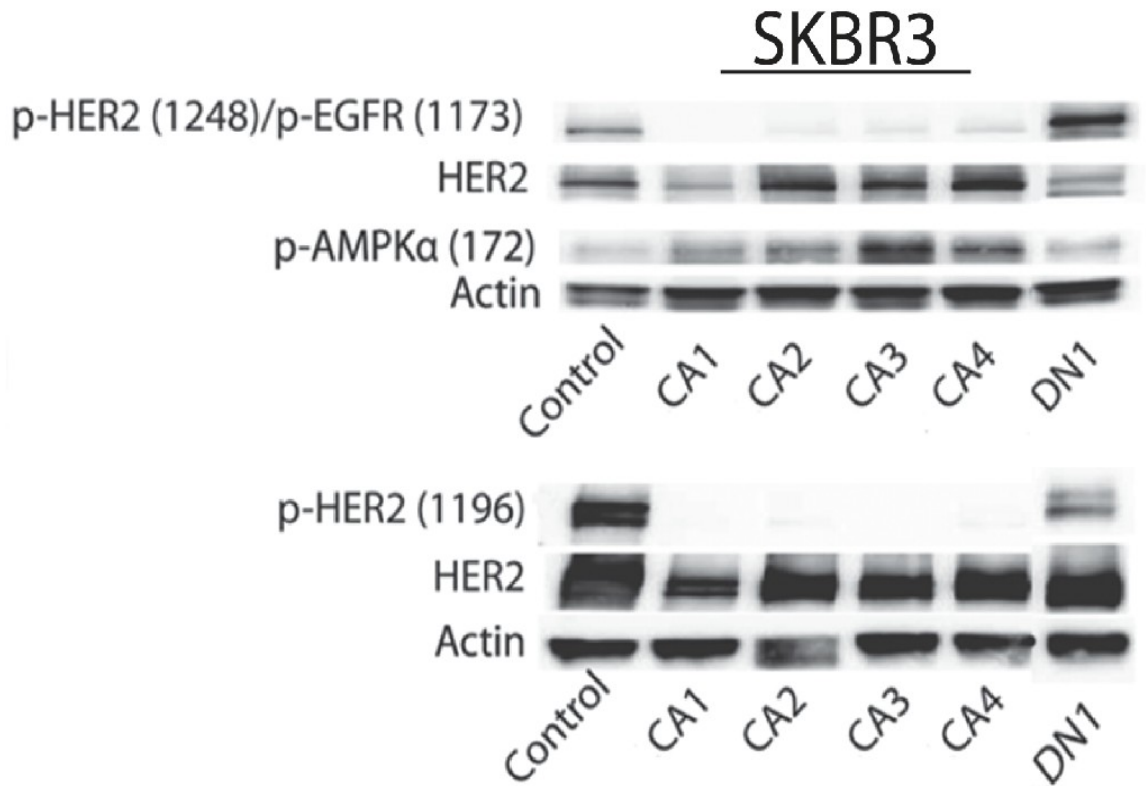


Figure 19: Regulatory role of AMPK in HER2 and EGFR activation is confirmed with AMPK Constitutively Active (CA) and Dominant Negative (DN) constructs in SKBR3. SKBR3 cells transfected with constitutively active (CA) AMPK α show decreased phosphorylation of HER2 (1248 and 1196) and EGFR (1173), whereas cells transfected with dominant negative (DN) AMPK α show increased phosphorylation of HER2 (1248) and EGFR (1173).

Figure 20

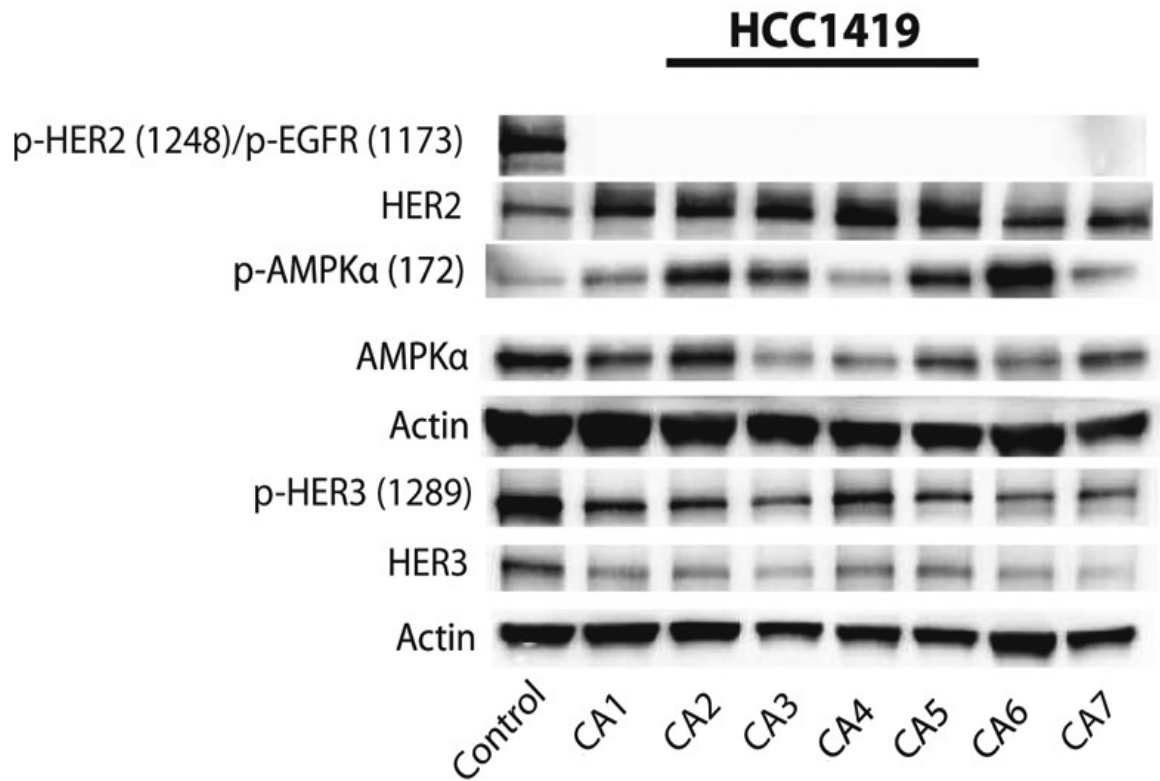


Figure 20: Regulatory role of AMPK in HER2 and EGFR activation is confirmed with AMPK Constitutively Active (CA) and Dominant Negative (DN) constructs in HCC1419. HCC1419 cells transfected with constitutively active (CA) AMPK α show decreased phosphorylation of HER2 (1248), HER3 (1289) and EGFR (1173).

Figure 21

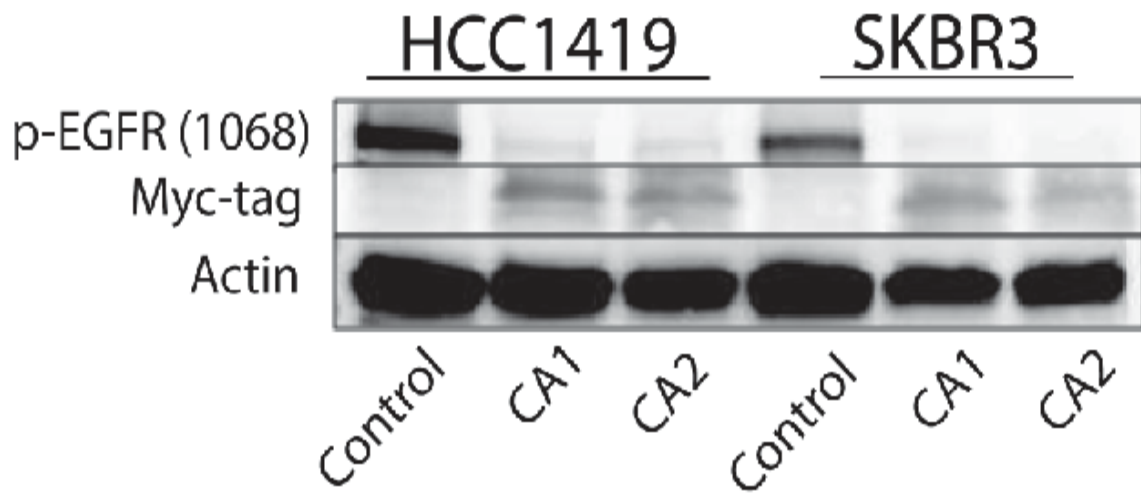


Figure 21: Constitutively Active AMPK regulates EGFR phosphorylation. In the HER2 positive breast cancer cell lines, HCC1419 and SKBR3, expression of a Constitutively Active AMPK construct negatively regulates the EGFR activating phosphorylation site (1068). Presence of a myc-tag protein indicates presence of the AMPK α CA construct.

Figure 22

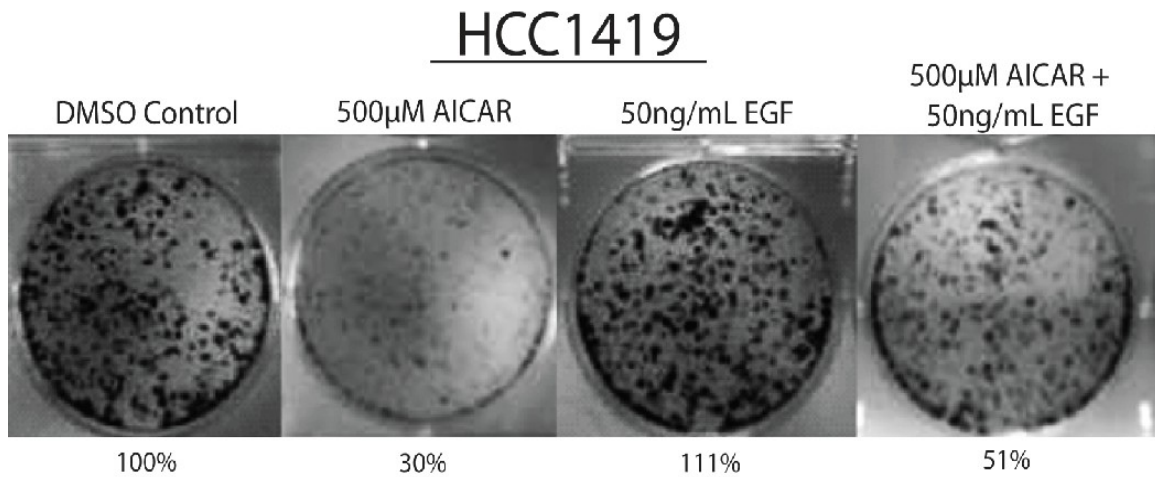


Figure 22: EGF treatment rescues AICAR-mediated growth inhibition in HCC1419 breast cancer. To test the importance of HER2/EGFR signaling in cell survival, we activated EGFR signaling with EGF ligand in the HCC1419 cell line. HCC1419 cells were treated with designated concentrations of AICAR and EGF. Experiments were conducted in triplicates. After two weeks, plates were stained with crystal violet. Staining was quantified by spectrophotometer-based absorbance analysis. Percentages shown below representative plates represent means of 3 independent treatments.

Figure 23

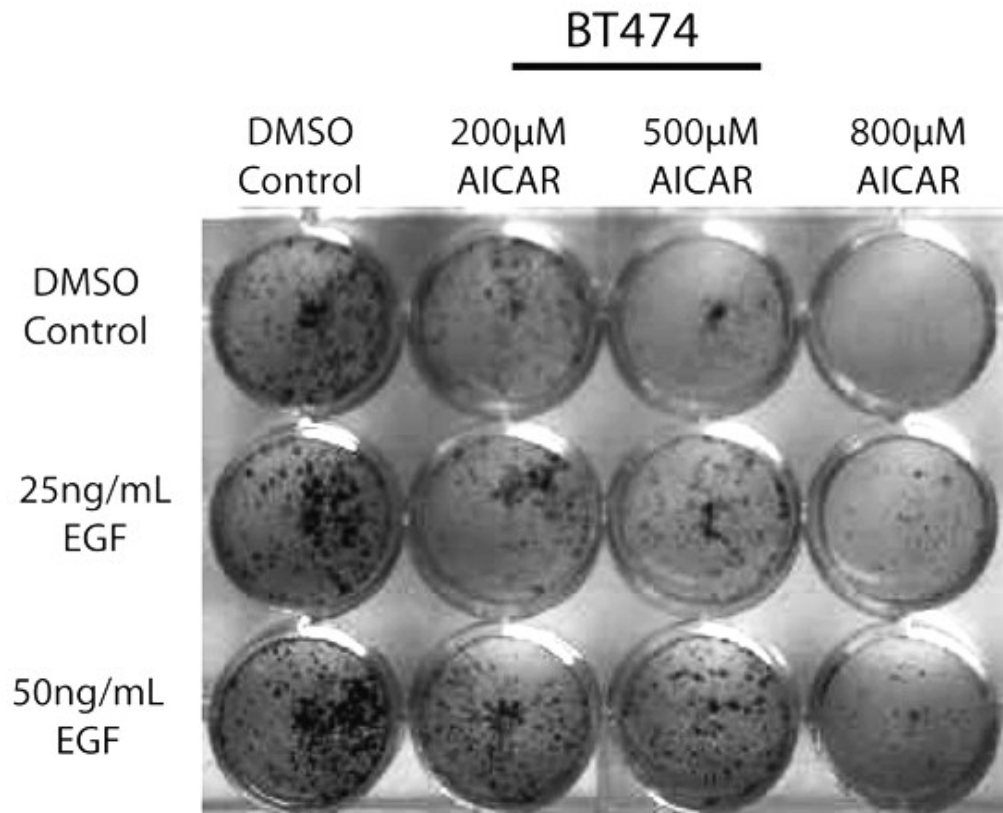


Figure 23: EGF treatment rescues AICAR-mediated growth inhibition in BT474

breast cancer. To test the importance of HER2/EGFR signaling in cell survival, we activated EGFR signaling with EGF ligand in the BT474 cell line. BT474 cells were treated with designated concentrations of AICAR and EGF. Experiments were conducted in triplicates. After two weeks, plates were stained with crystal violet.

Figure 24

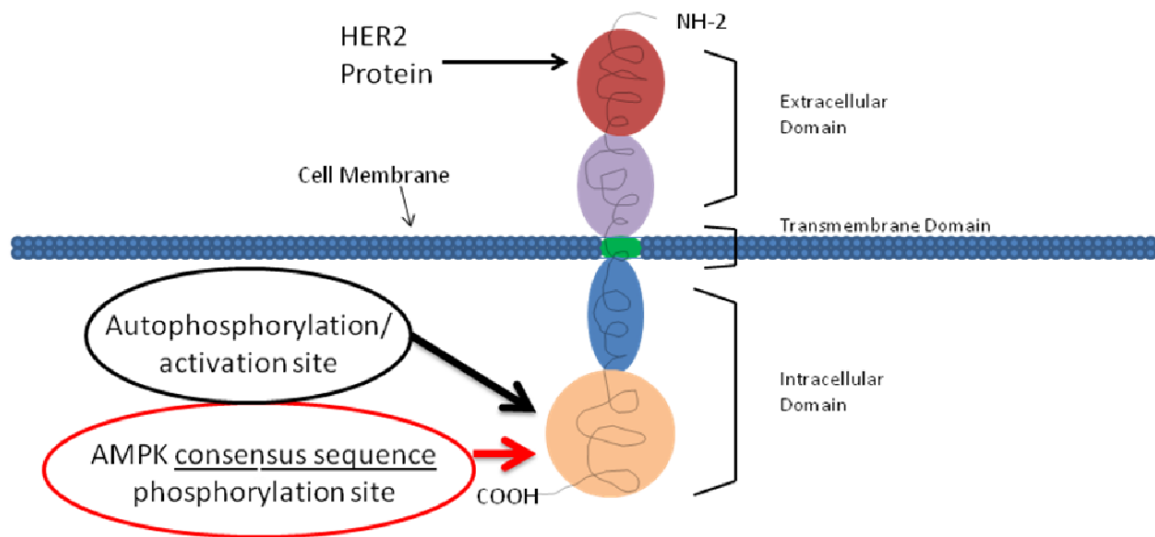
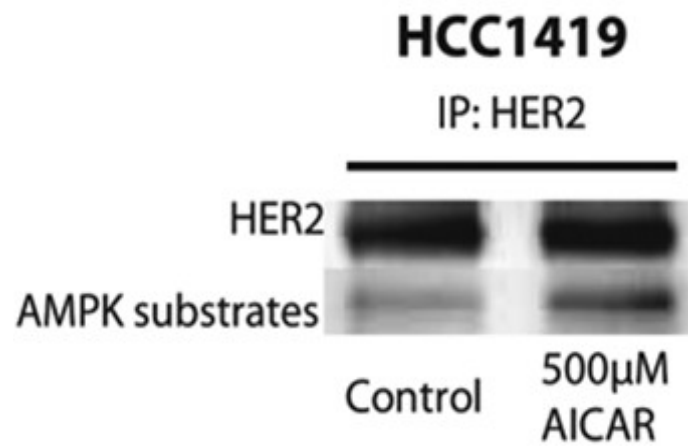


Figure 24: Identification of an AMPK substrate consensus sequence on HER2.

Diagram of HER2 protein with proposed AMPK substrate consensus sequence.

Figure 25

A



B

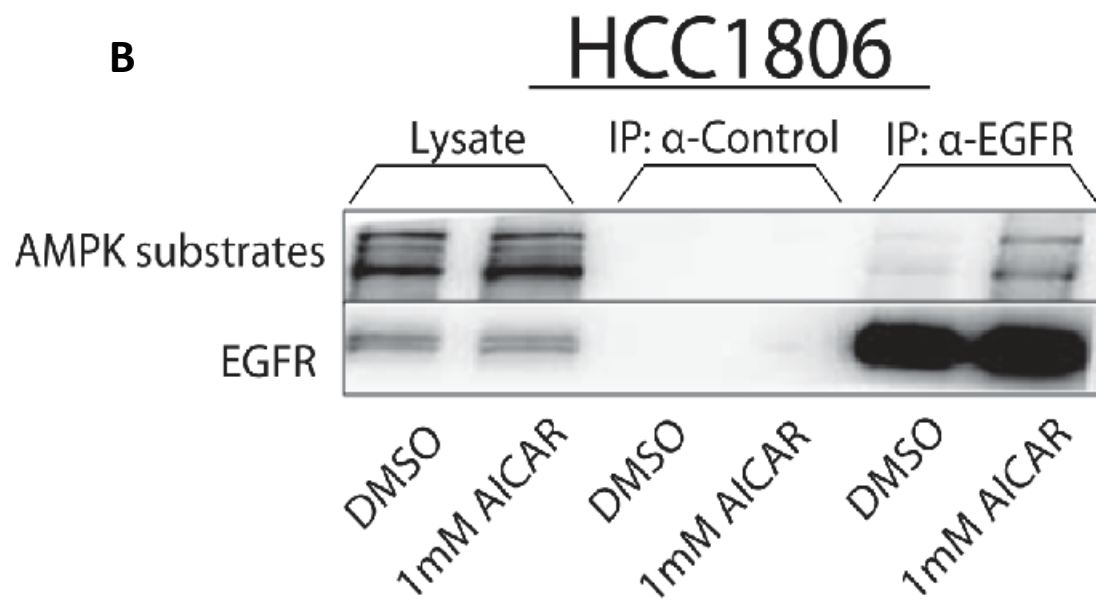


Figure 25: Activated AMPK phosphorylates an AMPK consensus site on HER2 and EGFR. Lysates of HCC1419 (A) and HCC1806 (B) cells treated with DMSO or AICAR for 24 hours were immunoprecipitated with anti-HER2 (HCC1419) or anti-EGFR (HCC1806) and probed with an antibody specific for phosphorylation of the AMPK consensus sequence (LXRXX(p-S/T)). Note increased phosphorylation of this AMPK consensus sequence on HER2 and EGFR proteins after AICAR treatment.

Figure 26

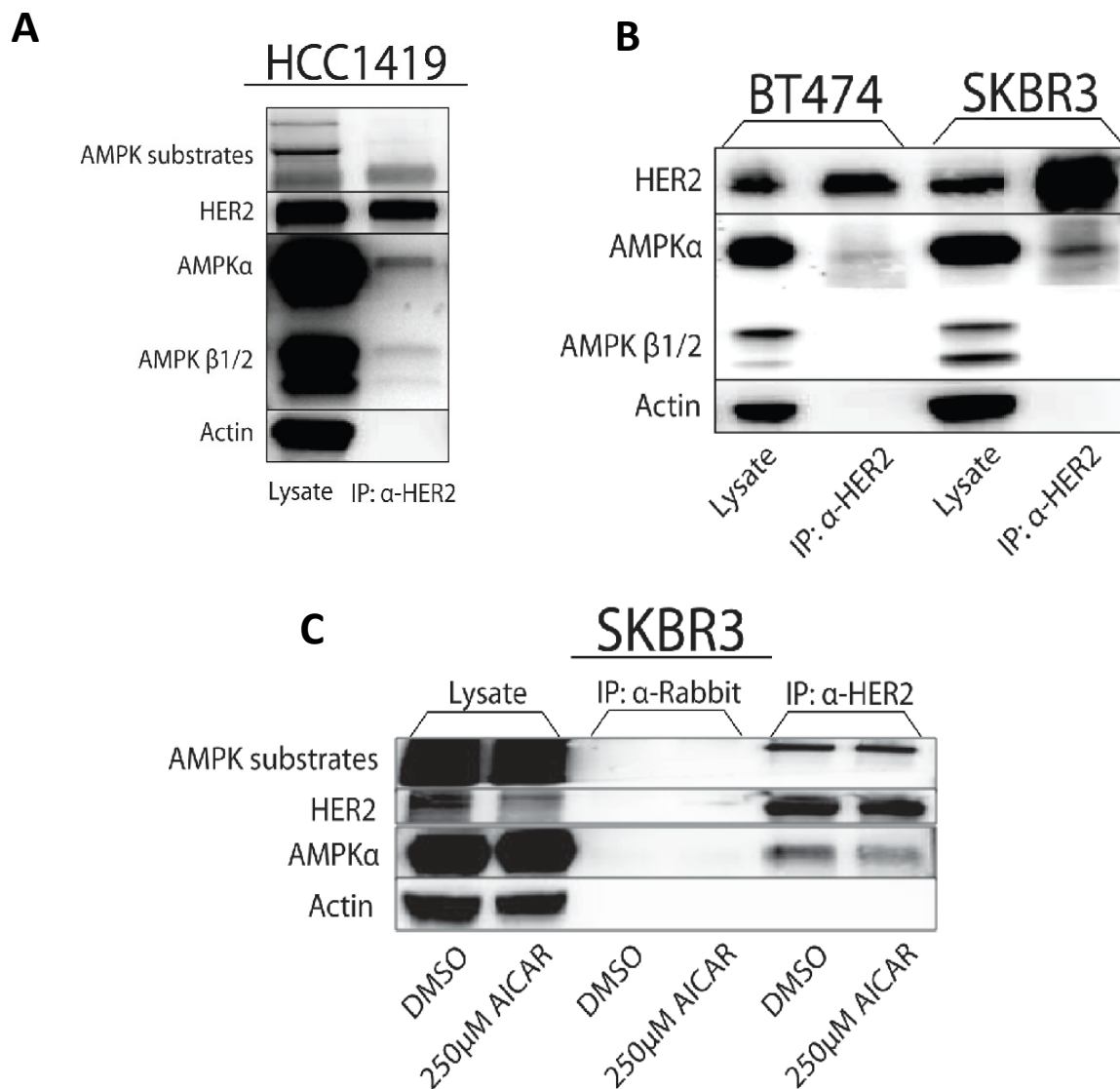


Figure 26: AMPK co-immunoprecipitates with HER2 in HER2-amplified breast cancer. HCC1419 (A), BT474 (B), and SKBR3 (B-C) cell lines were immunoprecipitated with anti-HER2. Western blot of HER2-immunoprecipitated lysates reveals AMPK binding to HER2.

Figure 27

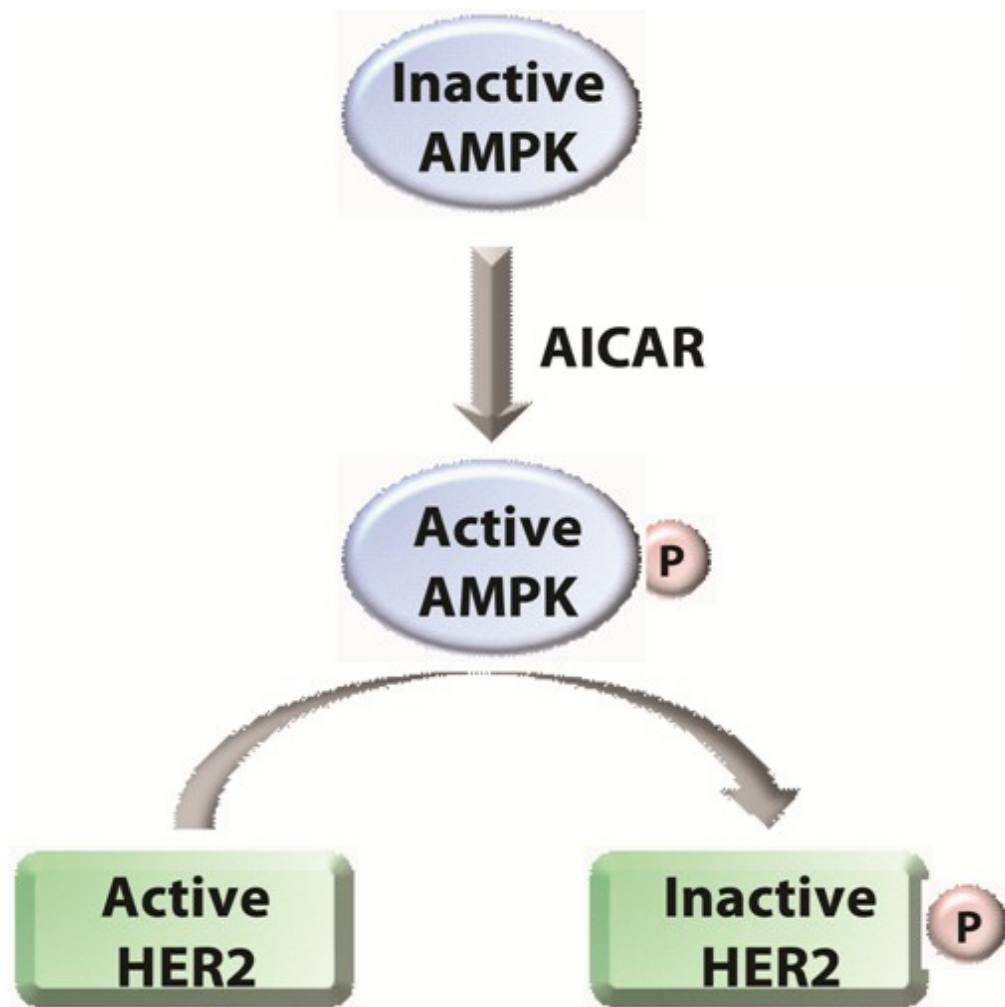


Figure 27: Proposed model of AMPK action on HER2.

Figure 28

AMPK substrate:	LXRXX*S/T
HER2a:	TLERPK <u>T</u> LSPG-RR (1172T)
HER2b:	SILRRRF <u>T</u> HQSDV-RR (900T)
HER2b ^{T→A} :	SILRRRF <u>A</u> HQSDV-RR
EGFRa:	FLQRYSDPTG-RR (1071S)
EGFRb:	SILHRIY <u>T</u> HQSDV-RR (892T)
EGFRb ^{T→A} :	SILHRIY <u>A</u> HQSDV-RR

Figure 28: Identification of potential AMPK phosphorylation sites on HER2 and EGFR. HER2 and EGFR amino acid sequences were analyzed for the AMPK substrate consensus sequence. We determined two potential phosphorylation sites per protein, designated HER2a, HER2b, EGFRa, and EGFRb. Mutant peptides (HER2b^{T→A}, EGFRb^{T→A}) were synthesized, where the proposed site of AMPK phosphorylation was changed to an alanine. These peptide sequences of HER2 and EGFR were utilized in the *in vitro* kinase assay.

Figure 29

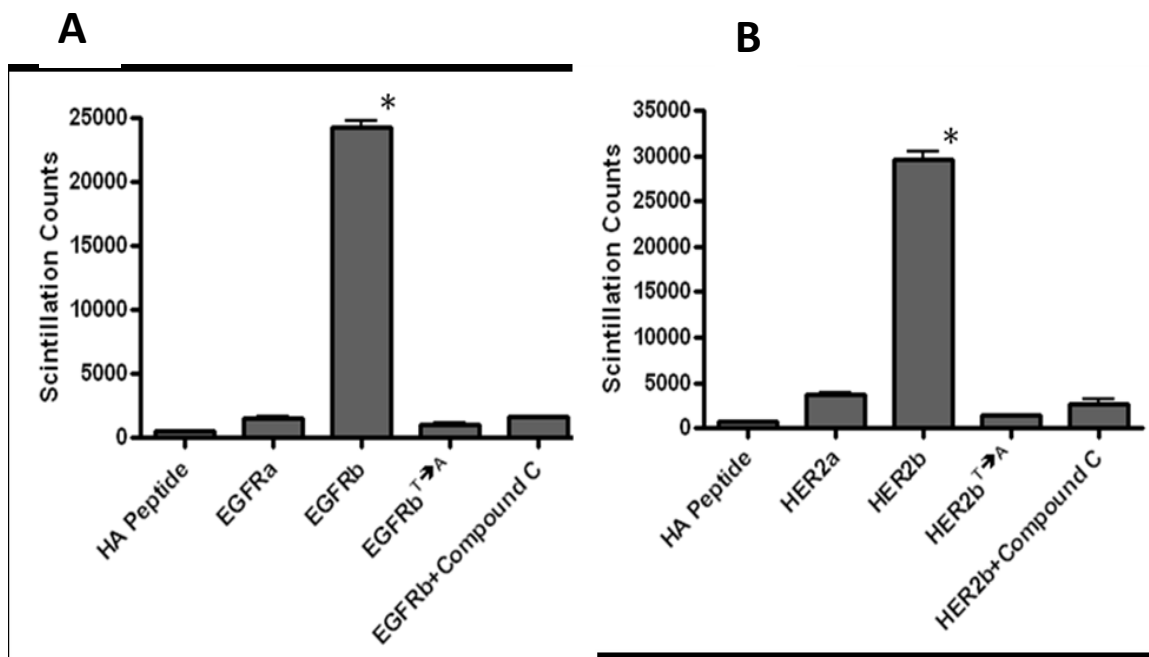


Figure 29: Activated AMPK phosphorylates an AMPK consensus site on HER2 and EGFR. (A) *In vitro* AMPK kinase assay using HER2 peptides (performed in triplicate) show AMPK-dependent phosphorylation of a specific HER2 sequence (HER2b, $p < 0.0002$). HA peptide was used as a negative control, Compound C was used as inhibitor of AMPK kinase activity, and a variant form (HER2b^{T→A}) of the peptide was used to demonstrate specificity of the phosphorylation site. (B) *In vitro* AMPK kinase assay using EGFR peptides (performed in triplicate) similarly shows AMPK-dependent phosphorylation of specific EGFR sequence (EGFRb, $p < 0.0001$). As above, compound C inhibits AMPK kinase activity, and a variant form (EGFRb^{T→A}) of the peptide, was used to demonstrate specificity of the phosphorylation site.

Chapter 4: AMPK is activated in Breast Cancer Cells by other Compounds, including Morin, a Natural Flavonoid

4.1 Introduction

AMPK activity regulates protein synthesis, glucose metabolism, and fatty acid synthesis [75, 84, 144, 146, 149, 168]. As such, AMPK activity must be tightly regulated during carcinogenesis. AMPK activity has been found to be downregulated in primary breast cancer [181]. Recently, attention has been brought to the use of AMPK agonists in the treatment of cancer [69, 179, 181]. AMPK, as the master metabolic regulator of the cell, is an attractive target in the treatment of the highly metabolic condition of cancer [67, 69, 164, 170]. Although pharmacological activation of AMPK has been reported to induce apoptosis and/or inhibit the growth of cancer in both *in vitro* and *in vivo* settings, the main pathways downstream of AMPK that mediate this anti-cancer activity remains to be conclusively defined [67, 70, 149, 161, 163]. The matter is further complicated by both the wide variety of compounds that activate AMPK, and the differing means of AMPK activation [69, 83, 90, 107, 113-116, 119, 141, 142, 150, 179].

In this section, we utilize pharmacological manipulation of AMPK to determine the differences and similarities in the mechanism of action of AMPK in breast cancer. First, we tested metformin, a biguanide used in the treatment of diabetes, that is known to activate AMPK in hepatocytes [101, 102, 104, 105, 141]. Metformin directly inhibits complex I of the respiratory chain, which affects the AMP:ATP ratio, which subsequently activates AMPK [95, 141]. Subsequently, we tested morin, a flavonoid, which was previously determined to inhibit fatty acid synthase (FASN) *in vitro* [137]. Additional studies attribute morin with diverse functions, including anti-tumorigenic activity [121-

123, 128, 129, 131, 135]. As AMPK activation is tied to inhibition of fatty acid synthase activity, and previous reports determined a variety of flavonoids can activate AMPK, we sought to determine if morin activates AMPK in breast cancer [22, 116, 119, 120]. C75, a synthetic FASN inhibitor, was shown to have anti-tumorigenic activity in breast cancer [24, 196]. As both morin and C75 are reported to inhibit FASN, we sought to determine if the two compounds have similar effects on the AMPK pathway. 2-Deoxy-D-glucose (2DG) is an analog of glucose that cannot be utilized to produce energy [197]. 2DG is known to activate AMPK by compromising the efficiency of energy produced through glucose metabolism [197, 198]. Through the use of several different AMPK activating compounds, we have attempted to clarify the mechanism behind AMPK-mediated anti-tumorigenic activity in breast cancer.

4.2 Results

4.2.1 AMPK-activating compounds decrease HER2+ breast cancer viability

An initial screen of AMPK-activating compounds on HER2+ breast cancer exhibited a range of results (Figure 30). HER2+ breast cancers demonstrated sensitivity to all AMPK-activating agents, although the concentration of the pharmacological agents varied from 100 μ M to 3mM. Compared to SKBR3, HCC1419 breast cancer cells displayed greater sensitivity to 2DG, which indicates a greater dependence on glucose metabolism in HCC1419 cells (Figure 30). Inhibition of cell viability by metformin was highly time dependent, as a 24 hour treatment resulted in a 20-40% decrease in cell viability, while a 72 hour treatment had a 40-60% decrease in cell viability (Figure 30). AICAR, the canonical activator of AMPK, was more effective in decreasing cell viability than either 2DG or metformin treatment (Figure 30). A 72 hour 1mM AICAR treatment

resulted in a 70-80% decrease in cell viability in HER2+ breast cancer. Morin treatment highly depended on concentration. The 100 μ M morin treatment hardly had any effect on cell viability, while the 200 μ M morin treatment had a profound effect on cell viability (Figure 30).

Colony forming assays of morin treatment in the HER2+ breast cancers SKBR3 and HCC1419, the EGFR+ breast cancers BT549 and MDA231, and the ER+ breast cancer MCF7, demonstrated a greater sensitivity to morin treatment in HER2+ and EGFR+ breast cancer (Figures 31-33). Treatment of HER2+ and EGFR+ breast cancers with 200 μ M morin greatly affected cell growth, while 200 μ M morin treatment in MCF7 barely affected cell growth (Figures 31-33).

Metformin treatment in EGFR+ breast cancer displayed results similar to morin (Figure 34). BT549 was sensitive to metformin at concentrations lower than MCF7. A treatment of 10mM metformin was necessary to prevent cell growth in MCF7, while 3mM treatment was sufficient to prevent growth of BT549 (Figure 34).

4.2.2 Metformin and morin treatment activate AMPK in breast cancer cell lines

Although metformin activates AMPK, its direct target is the mitochondrial complex I of the respiratory chain [95, 141]. By inhibiting the mitochondrial respiratory chain, metformin causes a drop in the rate of ATP synthesis [199]. Metformin's indirect activity on AMPK activation may explain its lower effectiveness in inhibiting cancer cell growth. Activated AMPK, shown by phosphorylation of the AMPK α site threonine 172, is observed after metformin treatment for 96 hours in breast cancer (Figure 35).

Contrastingly, 24 hour treatment with either 100 μ M or 200 μ M morin demonstrates a clear activation of AMPK in HER2+ breast cancer (Figure 36). Activation

of AMPK by morin is comparable to AICAR treatment (Figure 36). As morin was previously reported to be a potent inhibitor of FASN *in vitro*, we sought to determine if morin's effect on AMPK was related to inhibition of FASN [137]. However, treatment of BT474 with C75, a synthetic FASN inhibitor, did not activate AMPK (Figure 37). As C75-mediated inhibition of FASN did not activate AMPK, we determined that morin-induced activation of AMPK must occur separately from FASN inhibition.

4.2.3 Metformin and morin treatment inhibit the activation of EGFR in HER2+ breast cancer

Preliminary experiments looking at the effect of metformin treatment on HER2+ breast cancer demonstrate results similar to the effect of AICAR. Metformin treatment inhibited activation of EGFR in HER2+ breast cancer (Figure 38). Furthermore, morin treatment also displayed inhibitory effects on EGFR phosphorylation (Figure 39). Analysis of morin treatment on MAPK pathways revealed no effect on ERK1/2 phosphorylation; however, phosphorylation of RSK appeared to increase (Figure 40). Further investigation appears necessary to elucidate the exact impact of morin treatment on breast cancer. Interestingly, both metformin and C75 treatment inhibit mTOR phosphorylation, which might play a role in mediating the anti-tumor effects of these agents (Figure 41).

4.2.4 Morin and the level of cAMP in breast cancer

Previous studies have documented an existing relationship between cyclic adenosine monophosphate (cAMP) and adenosine monophosphate-activated protein kinase (AMPK). Although the scientific community appears unresolved as to the exact nature of this association between cAMP and AMPK, the general consensus provides that

an increase in cAMP levels leads to an early transient activation of AMPK. Prior studies also document a feedback mechanism that is responsible for inhibition of AMPK activity under conditions of continually elevated cAMP levels.

In the article “Resveratrol Ameliorates Aging-Related Metabolic Phenotypes by Inhibiting cAMP Phosphodiesterases,” the authors examine the interaction of the cAMP and AMPK pathways in C2C12 myotubes treated with resveratrol [112]. Treatment of mice with resveratrol, a polyphenol, is reported to delay aging-related phenotypes [112]. The beneficial effects of resveratrol on aging-related conditions are linked to the proteins sirtuin 1 (Sirt1) and sirtuin 2 (Sirt2) [109, 110, 171]. According to Park SJ, et al., resveratrol treatment causes an increase in cAMP levels through the inhibition of phosphodiesterases [112]. Intracellular levels of cAMP depend on two classes of proteins: adenylate cyclase and phosphodiesterases [112]. Adenylate cyclase synthesizes cAMP from ATP while phosphodiesterases hydrolyze cAMP to AMP [112]. By inhibiting phosphodiesterase, resveratrol prevents the breakdown of cAMP to AMP [112]. The subsequent increase in cAMP levels activates the cAMP-regulated guanine nucleotide exchange factors (cAMP GEFs) Epac1 and Epac2 [112]. Epac1 is required for resveratrol-mediated activation of AMPK. Epac1 activates AMPK indirectly by increasing intracellular Ca^{2+} levels, leading to the activation of calcium/calmodulin-dependent kinase kinase β (CamKK β), a known AMPK kinase [200-203].

We have shown that morin, a flavonoid, also activates AMPK in cell culture conditions. However, the mechanism behind morin-mediated activation of AMPK remains undetermined. To ascertain whether morin activates AMPK through modulation of cAMP levels, we measured cAMP levels in breast cancer cell cultures under treatment

conditions (Figures 42 and 43). Based on the results, it appears that cAMP levels fluctuate within the cell regardless of treatment (Figures 42 and 43). For BT474, a HER2+, ER+ breast cancer cell line with a PIK3CA mutation, a 24 hour AICAR treatment slightly increased cAMP levels. In HCC1419, 24 hour treatment of 2-deoxy-D-glucose or morin caused an increase in cAMP levels. A 2 hour treatment of 2,5 dideoxyadenosine (adenylate cyclase inhibitor) or lapatinib (HER2/EGFR inhibitor) also effectively increase levels of cAMP. Contrastingly, a 2 hour treatment of HCC1419 with EGF decreases overall cAMP levels. A 24 hour treatment of HCC1419 with 500 μ M AICAR increases cAMP levels slightly, while a 24 hour treatment of 1mM AICAR decreases cAMP levels. As cAMP is a second messenger, its role within the cell is transient. Due to the large variability in cAMP levels, we cannot conclusively state that AMPK activation occurs through inhibition of phosphodiesterases in morin-treated cells. Future studies that focus on correlating morin-based activation of AMPK with the inhibition of phosphodiesterases should also measure the level of intracellular Ca^{2+} and activation of CamKK β .

4.3 Discussion

In summary, we have shown that AMPK activating agents preferentially inhibit the growth of HER2+ breast cancer. Metformin, a biguanide currently used in the clinic to treat diabetes, required extended treatment time points to activate AMPK. Metformin activates AMPK indirectly, which may explain the amount of time required for AMPK activation. Morin, a flavonoid, has been attributed with many functions. Morin is reported to inhibit fatty acid synthase (FASN), inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), and the inflammatory response [122, 124, 131, 137]. Here,

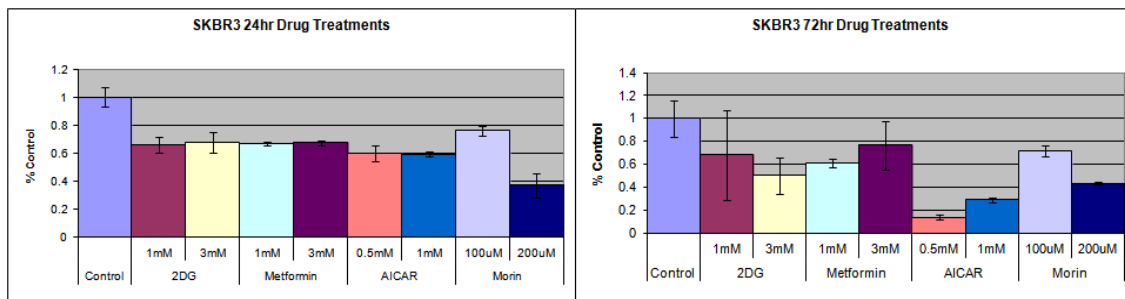
we describe a novel function for morin in the activation of AMPK. Contrastingly, C75, a known FASN inhibitor, does not activate AMPK, indicating that morin activity on AMPK cannot be attributed to FASN inhibition. Correspondingly, lack of C75-based activation of AMPK has been observed in prior published articles [204]. Furthermore, similar to AICAR activity, both metformin and morin have inhibitory effects on EGFR activation. We propose the enhanced sensitivity of HER2+ breast cancers to AMPK-activating agents is through AMPK-mediated inhibition of the ErbB family of growth factor receptors.

HER2+ breast cancer is highly dependent on HER2/EGFR signaling. In this study, we describe a novel mechanism wherein AMPK directly mediates an inhibitory effect on HER2 and EGFR signaling. While several studies have focused on uncovering AMPK substrates, HER2 and EGFR represent previously unstudied substrates of AMPK. In Banko et al., the use of HEK293T cells in their screen for AMPK substrates overlooked HER2 and EGFR because HEK293T cells do not express either HER2 or EGFR [205-207]. By using the pharmacological agents- metformin, AICAR, and morin, we demonstrate that inhibition of HER2/EGFR is through AMPK activation. Although the mechanism of AMPK activation through morin is currently unknown, we hypothesize morin activation of AMPK occurs in a mechanism that differs from resveratrol. Morin does not seem to have inhibitory activity on phosphodiesterases [112]. We propose the use of AMPK activating agents would be useful in the clinic for therapy of HER2 or EGFR-dependent cancers. Several types of cancer are dependent on either EGFR or HER2 signaling. HER2 signaling is important, not only in breast cancer, but also colon, ovarian, endometrial, lung, uterine cervix, head and neck, gastric and esophageal

carcinomas [41, 42, 49, 186]. EGFR signaling is equally important and found in a variety of cancer types including: breast, lung, glioblastoma, and squamous cell carcinomas [56, 61-65, 187]. Our results support the development of potent, clinically relevant AMPK activators as a novel therapeutic strategy for EGFR and HER2-dependent cancers.

Figure 30

A



B

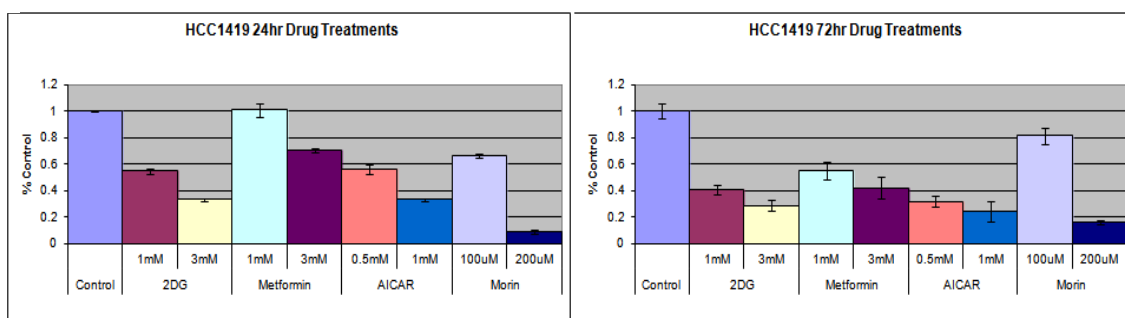


Figure 30: AMPK-activating agents reduce the survival of HER2-amplified breast cancer cell lines. MTT assay analysis of breast cancer cell line viability with DMSO control, 1mM and 3mM 2DG, 1mM and 3mM metformin, 0.5mM and 1mM AICAR, and 100μM and 200μM morin treatment. Breast cancer cell lines were seeded into a 96-well plate and treated for either 24 or 72 hours. Treatments were done in triplicates and results were measured using a spectrophotometer at 570nm with a reference wavelength of 690nm. Error bars indicate SD.

Figure 31

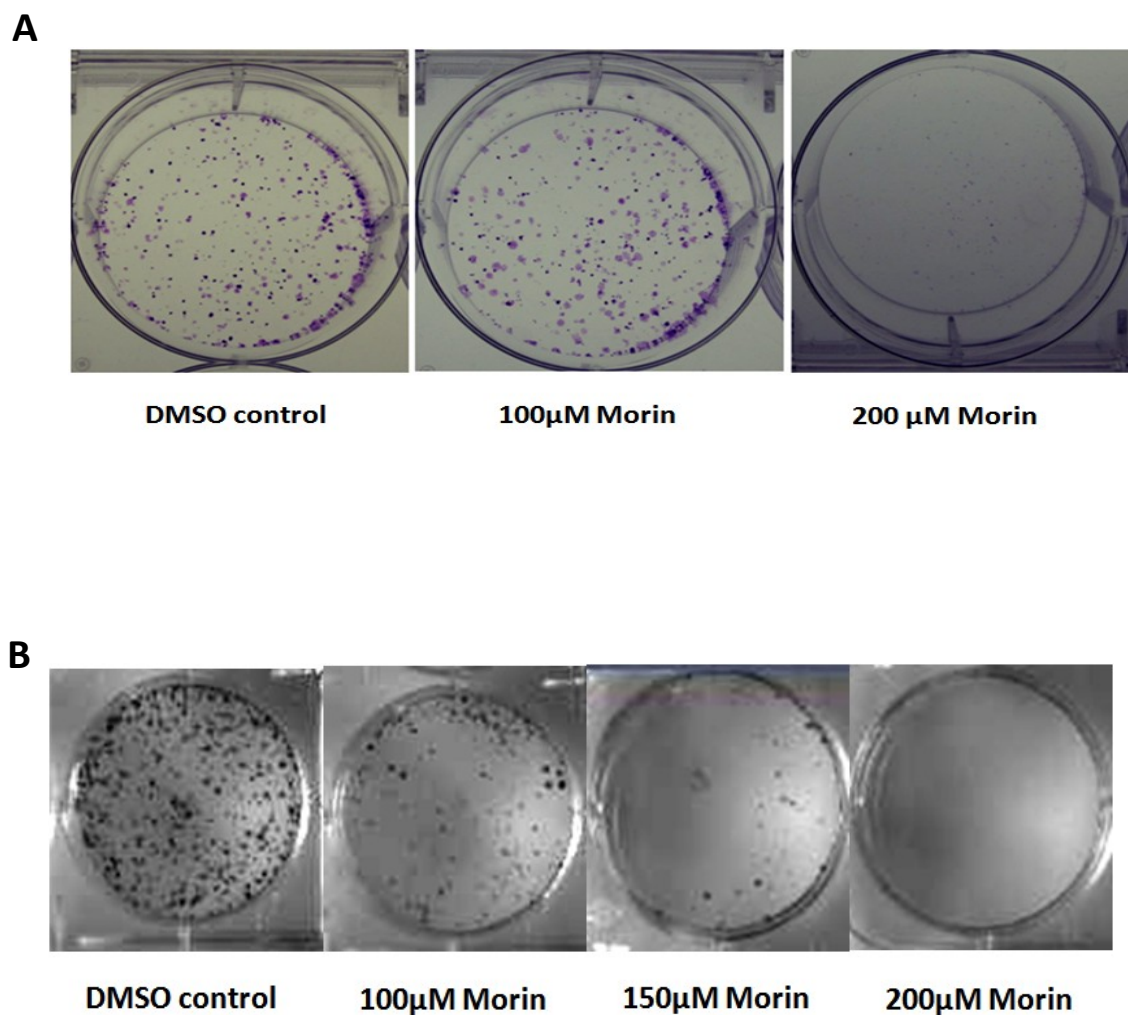


Figure 31: Cell growth inhibition in HER2+ breast cancer cell lines treated with morin. Clonogenicity of HCC1419 (A) and SKBR3 (B) cells with treatment of the indicated amounts of morin. Cells were treated for 2 weeks then fixed and stained. Colony forming assays were done in triplicate.

Figure 32

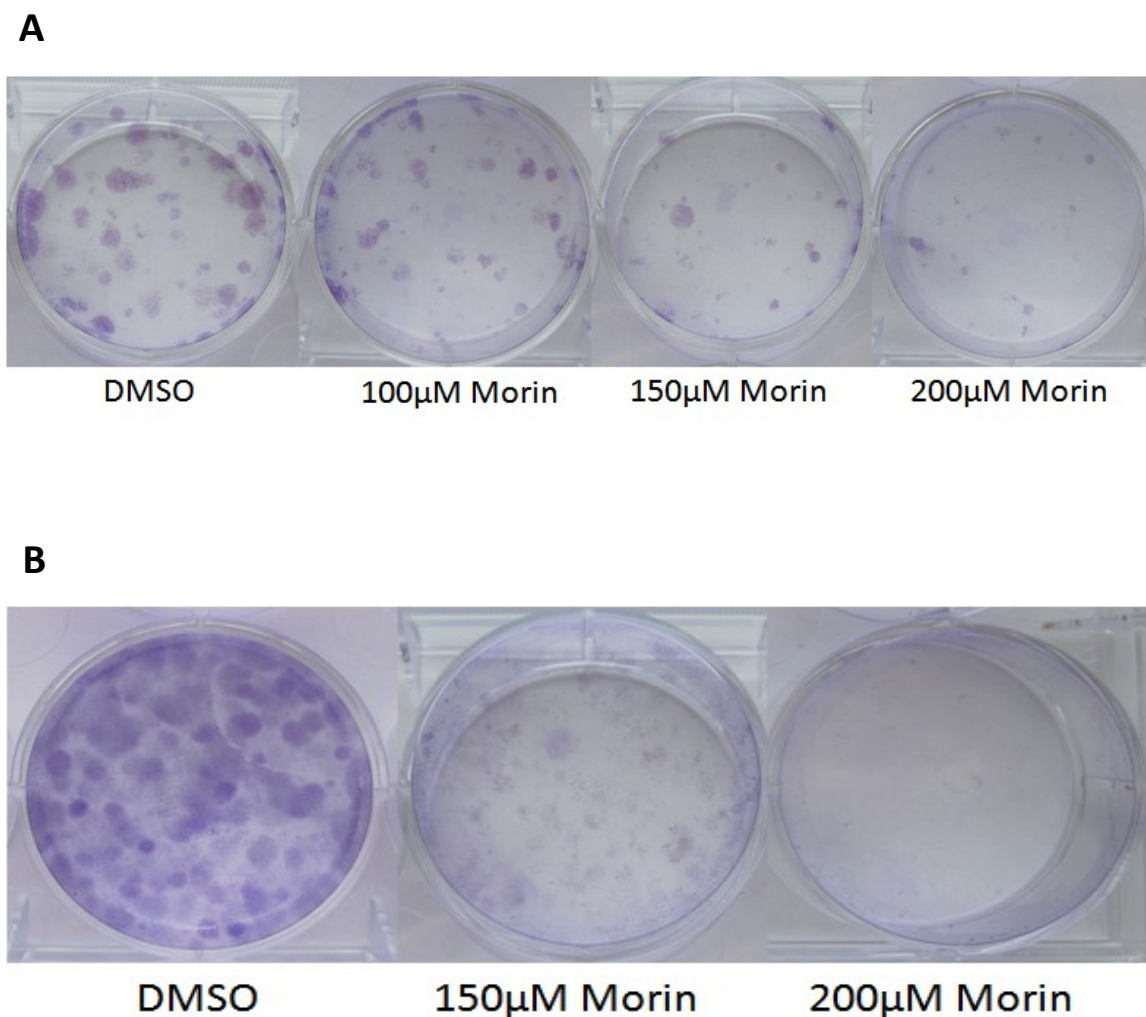


Figure 32: Cell growth inhibition in EGFR+ breast cancer cell lines treated with morin. Clonogenicity of BT549 (A) and MDA231 (B) cells with treatment of the indicated amounts of morin. Cells were treated for 2 weeks then fixed and stained. Colony forming assays were done in triplicate.

Figure 33

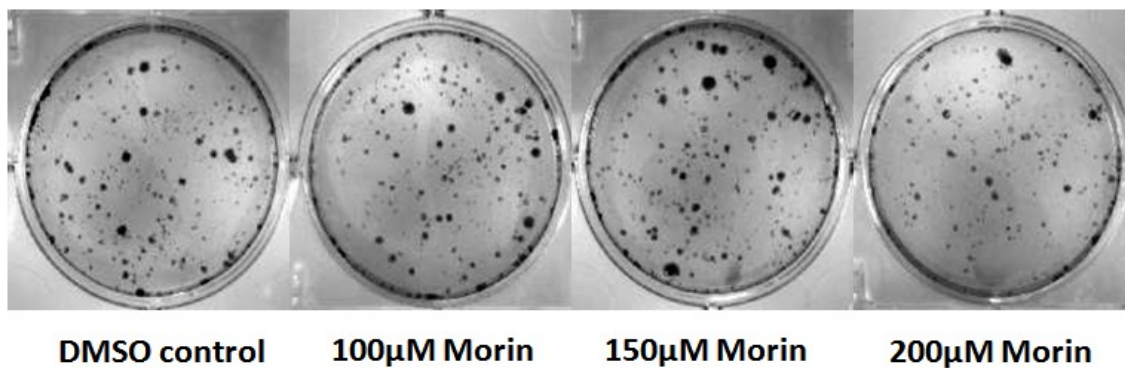
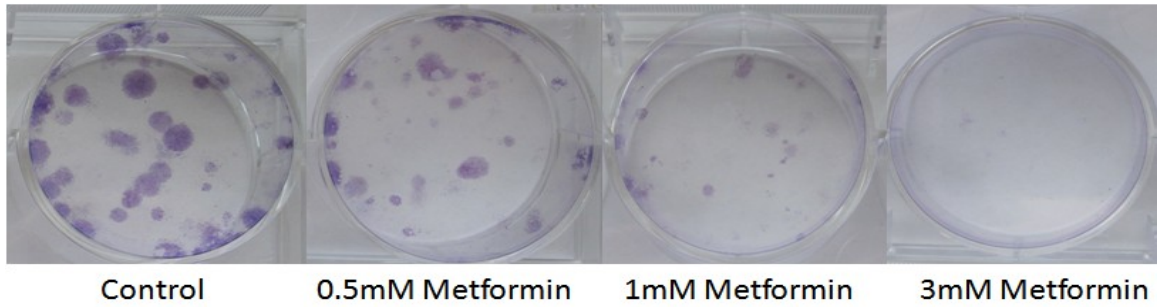


Figure 33: Morin treatment in ER+ breast cancer does not affect cell viability.

Clonogenicity of MCF7 cells with treatment of the indicated amounts of morin. Cells were treated for 2 weeks then fixed and stained. Colony forming assays were done in triplicate.

Figure 34

A



B

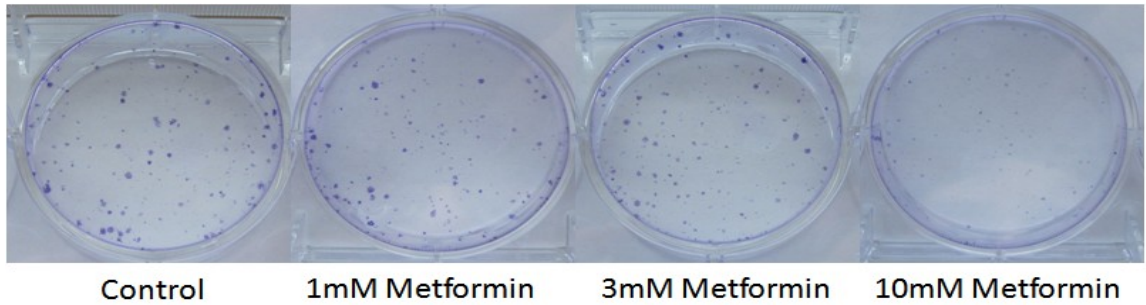


Figure 34: EGFR-dependent cell line, BT549, displays heightened sensitivity to metformin treatment. BT549 (A) and MCF7 (B) were treated with the indicated concentrations of metformin. Treatments were performed in triplicates. Note the higher concentration of metformin required to inhibit cell growth.

Figure 35

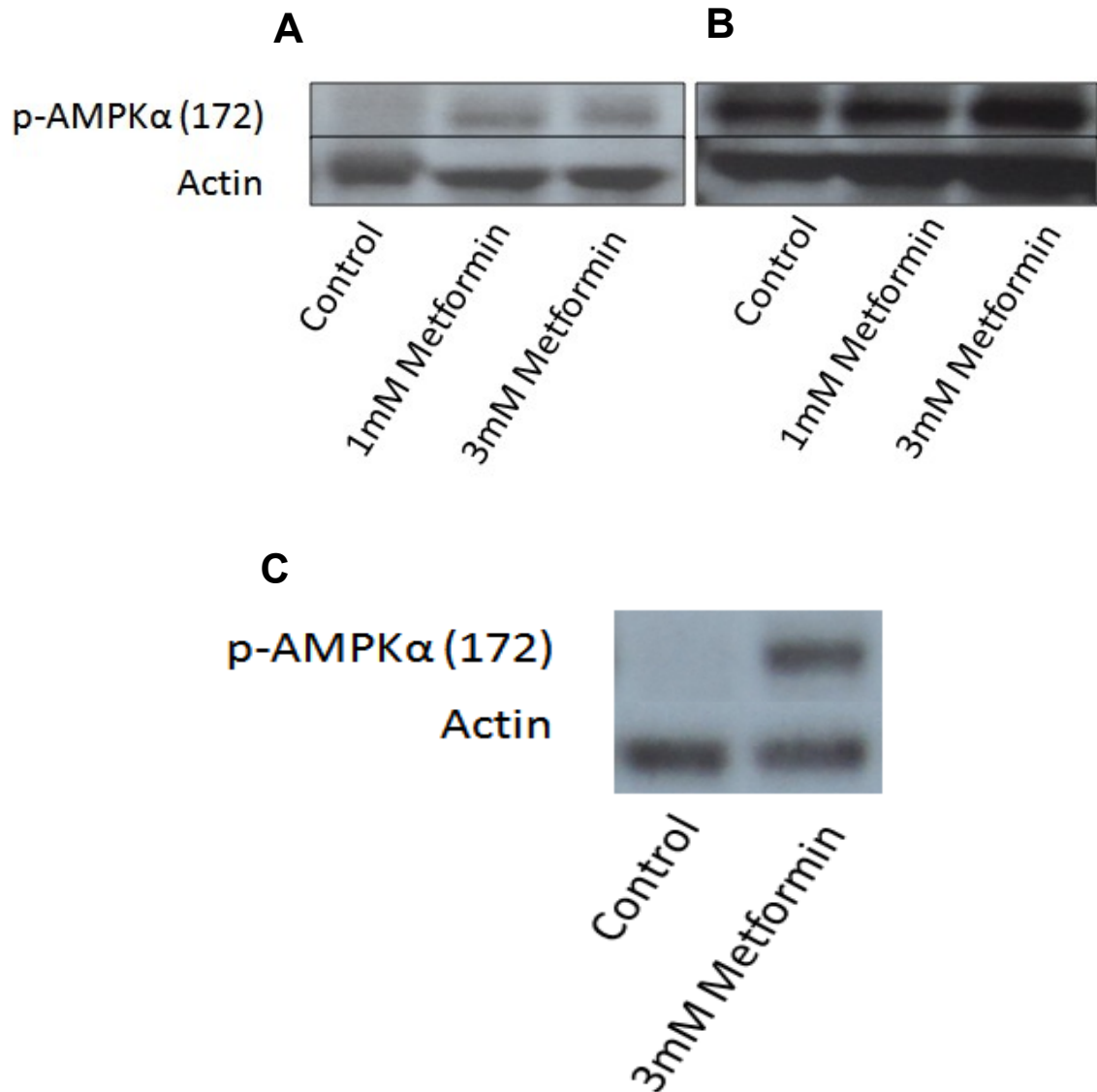
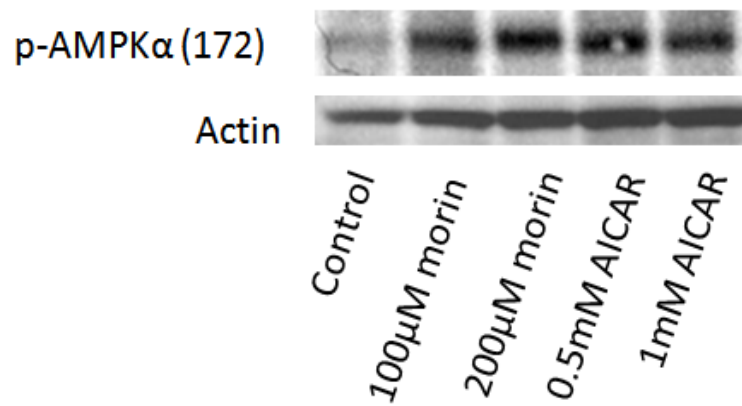


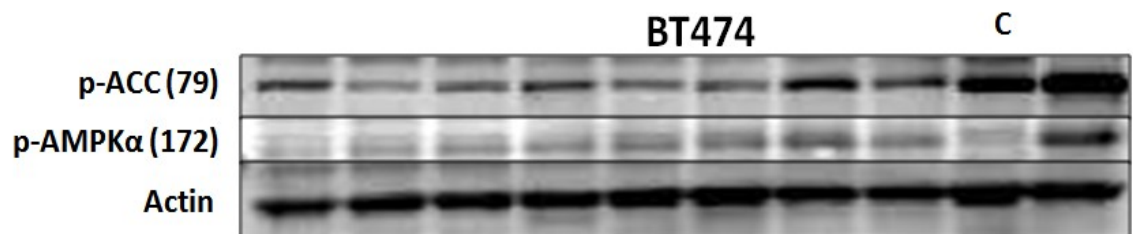
Figure 35: Metformin treatment activates AMPK in HER2+ breast cancer. Western blot analysis of 96 hour metformin treatment in the MCF10A (A), and the HER2 positive cell lines BT474 (B) and SKBR3 (C).

Figure 36

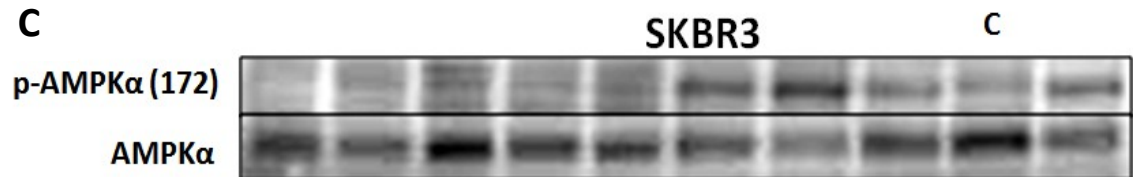
A



B



C



D

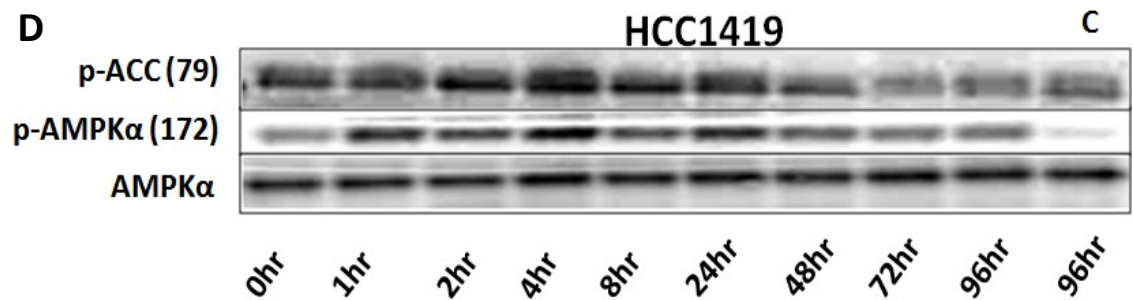


Figure 36: Morin treatment activates AMPK in HER2+ breast cancer. Western blot analysis of 24 hour metformin treatment in HCC1419 (A), and time course based treatments of morin in BT474 (B), SKBR3 (C), and HCC1410 (D). Note how morin treatment activates AMPK in levels comparable to AICAR in HCC1419. The letter C represents the DMSO control treatment at the 96 hour time point.

Figure 37

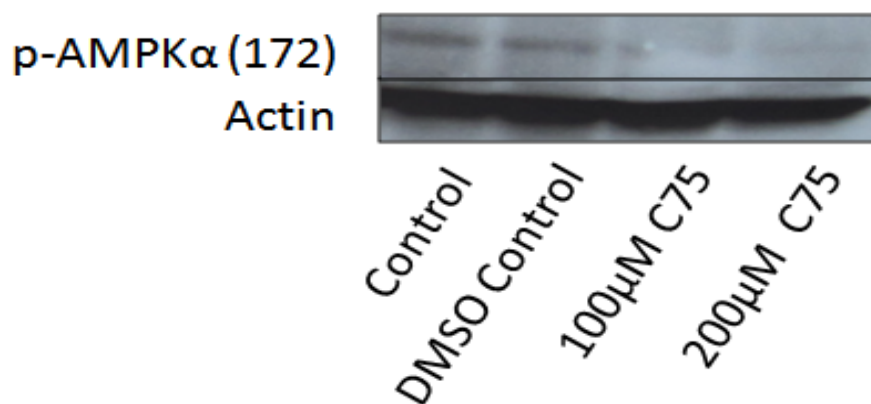


Figure 37: Inhibition of FASN does not activate AMPK. BT474 breast cancer cells were treated for 24 hours with the indicated concentrations of agents. Note that C75 treatment does not activate AMPK.

Figure 38

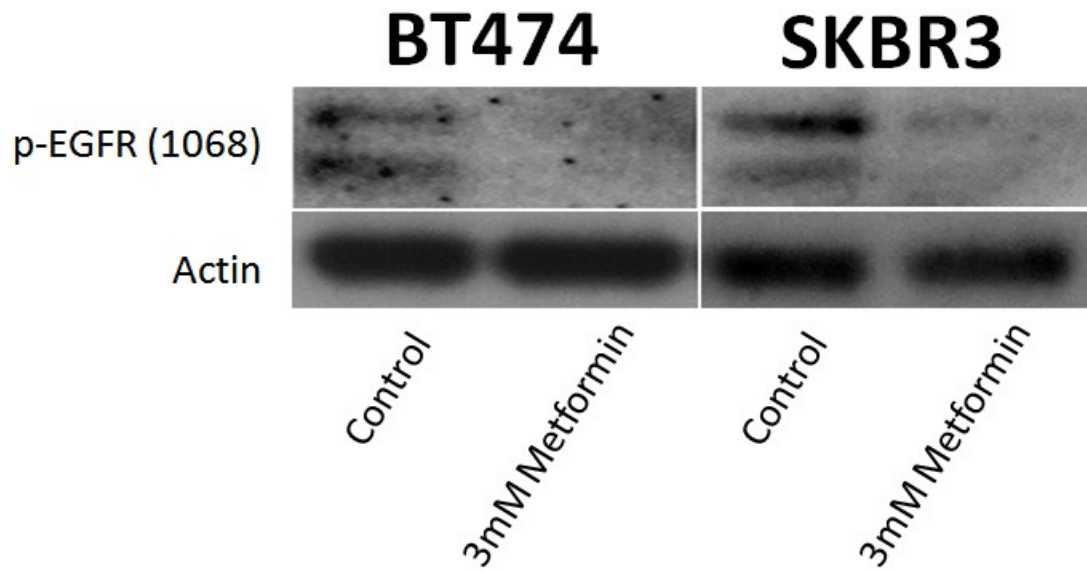


Figure 38: Metformin treatment inhibits EGFR activation. BT474 and SKBR3 cells were treated with either control or 3mM metformin for 96 hours. In HER2+ breast cancer, activation of EGFR decreases with continuous metformin treatment.

Figure 39

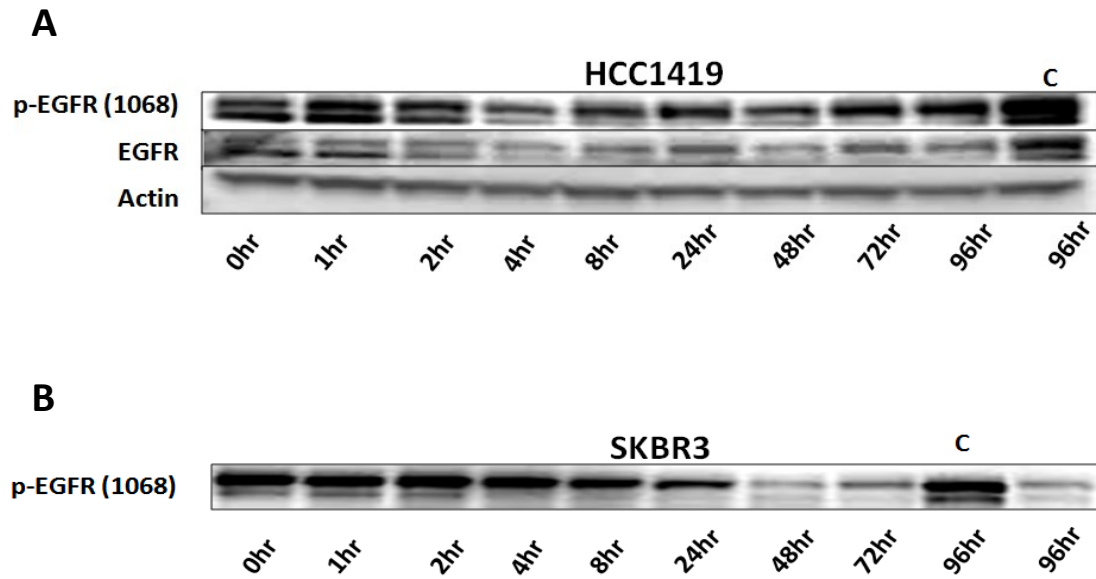


Figure 39: Morin treatment inhibits EGFR activation in HER2+ breast cancer.
HCC1419 (A) and SKBR3 (B) were treated with 200 μ M morin for the indicated time points. Note decreased phosphorylation of EGFR with continuous treatment of morin.

Figure 40

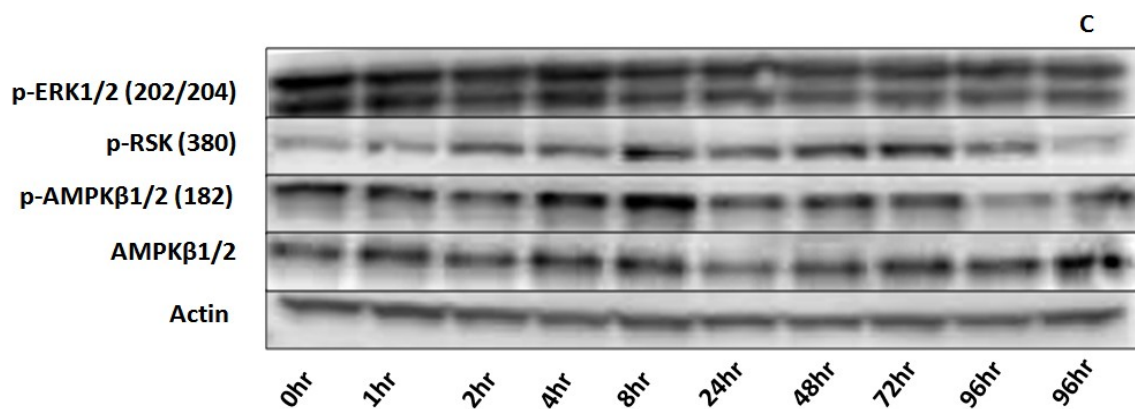


Figure 40: Morin does not inhibit ERK1/2 signaling. HCC1419 treated with 200μM morin for the indicated time points. The letter C represents control DMSO treatment at 96 hours. Note p-ERK1/2 levels remain stable; however, levels of p-RSK appear to increase. Given that RSK phosphorylation is directly downstream of ERK, the cause of the above seen increase in p-RSK levels is currently unknown.

Figure 41

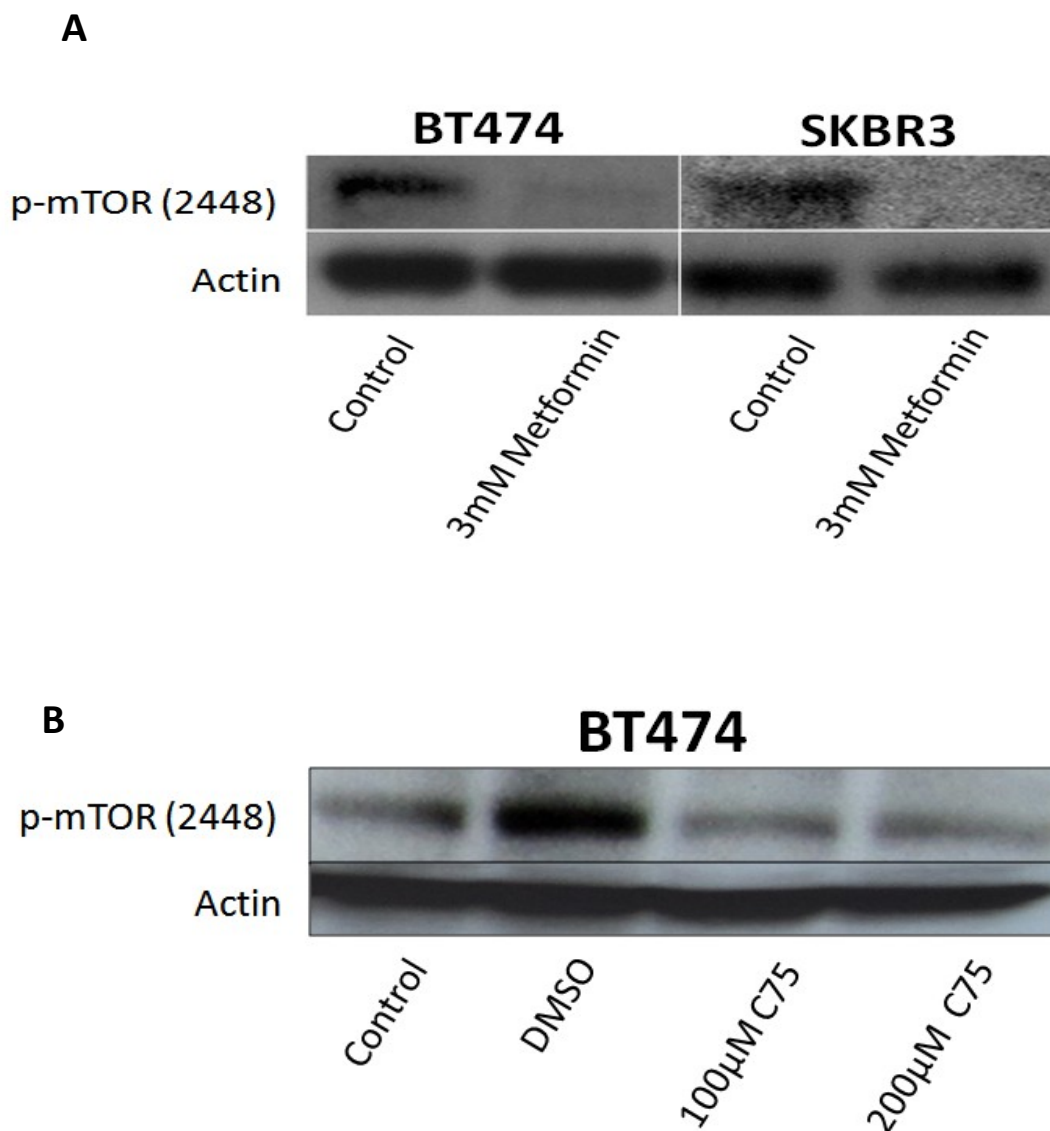


Figure 41: Treatment of metformin or C75 inhibit mTOR activation. BT474 and SKBR3 cells (A) were treated for 96 hours with 3mM metformin. BT474 (B) was treated for 24 hours with the indicated concentrations of the FASN inhibitor, C75. Levels of p-mTOR (2448) were determined by Western blot.

Figure 42

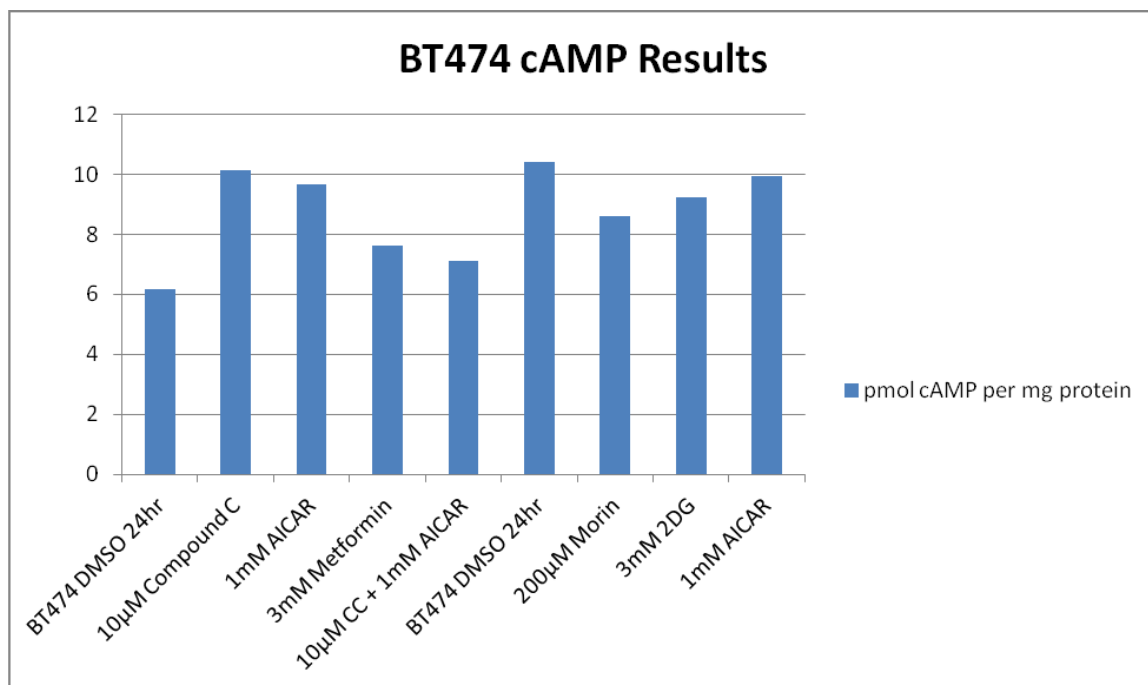


Figure 42: Measurement of cAMP levels in BT474 following pharmacological treatment. BT474 was treated for 24 hours with the following: DMSO (control), Compound C, AICAR, metformin, morin or 2DG at the indicated concentrations. Results are shown as pmol cAMP/mg protein. Note that in the first course of treatments, Compound C, an AMPK inhibitor, and AICAR caused a slight increase in the levels of cAMP. In the second course of treatments, none of the pharmacological agents increased the level of cAMP.

Figure 43

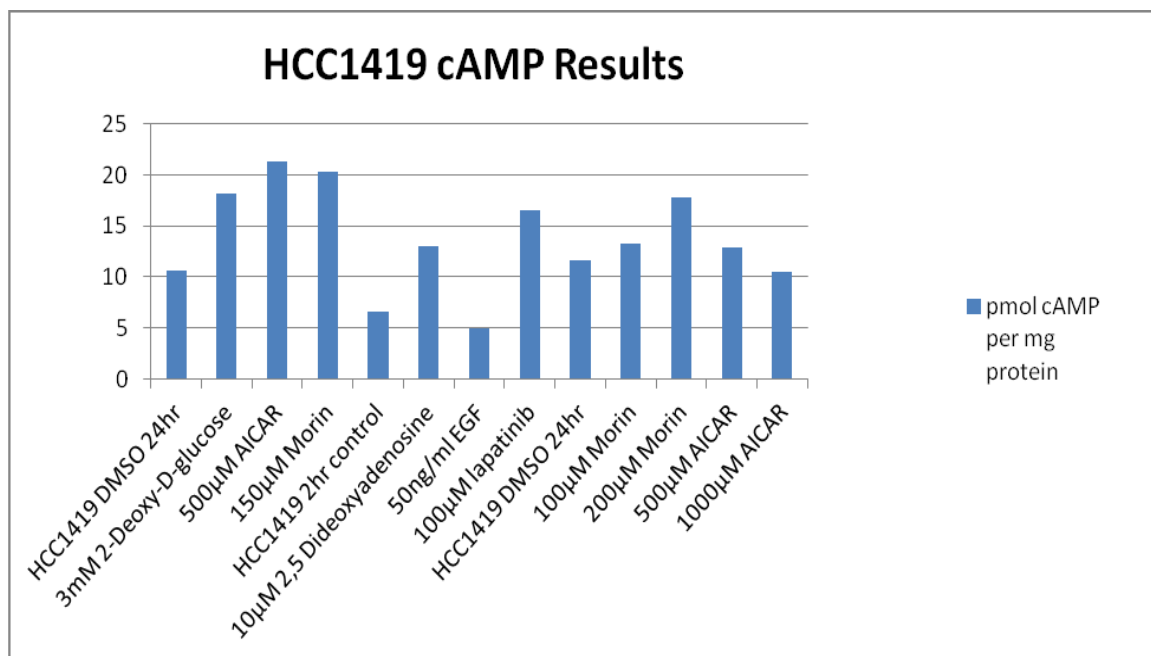


Figure 43: cAMP measurement in HCC1419 breast cancer cells following pharmacological treatment. HCC1419 cells were either treated for 2 or 24 hours. The 2 hour treatments involved: 2,5, dideoxyadenosine, EGF or lapatinib. The 24 hour treatments included: 2-deoxy-D-glucose, AICAR, and morin. Results are shown as pmol cAMP/mg protein. Note the large variation in cAMP levels, regardless of treatment. Further investigation into this area is required for conclusive results.

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AWARDS

Johns Hopkins University School of Medicine, Baltimore, MD

Pathology Young Investigator’s Award (Excellence in basic research)

2013

Graduate Student Association Travel Award (Top 5% of applicants)

2013

Student Recognition Award (Nomination-based award for leadership excellence)

2011

University of California, Riverside

CNAS Dean’s Summer Internship Program (Awarded to top 10% of applicants)

2006

NOVA Grant (Awarded to 3 female students for science-based research excellence)

2005

Governor’s Scholar Award (Top 5% of students)

2004

UCR Regent’s Scholarship (Top 1% of applicants)

2004

LEADERSHIP EXPERIENCE

Johns Hopkins School of Medicine, Baltimore, MD

Director of Finance

2012-Present

Student Ambassador Program (SAP)

Organized 6 annual cultural events aimed at celebrating the cultural diversity of the Hopkins graduate student body.

Peer Mentor

2011-Present

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Spearheaded seminars that address graduate student concerns.

Seminar topics ranged from choosing a thesis lab to preparing for the graduate qualifying examination.

PROFESSIONAL EXPERIENCE

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Graduate Student

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Principal Investigator: Dr. Edward W. Gabrielson

Designed and conducted genetic and pharmacological experiments to analyze the effect of AMPK activation on HER2 and EGFR phosphorylation in HER2 and EGFR-dependent breast cancer.

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Undergraduate Research Assistant

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Explored the role of the jasmonic acid and ethylene signaling pathways in the Mi-1 mediated defense response of the tomato plant.

PUBLICATIONS AND PAPERS

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POSTERS

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Jhaveri TZ, Woo JH, and Gabrielson E. “Targeting Metabolic Pathways in HER2-positive Breast Cancer.” Annual Safeway Breast Cancer Retreat. (2012) Baltimore, MD.

TALKS

Jhaveri TZ, Woo JH, and Gabrielson E. “AMPK Regulates HER2/EGFR Activity in HER2-Positive Breast Cancer.” Pathobiology Retreat. (2013) Mt. Washington Conference Center, Baltimore, MD.

Jhaveri TZ, Woo JH, and Gabrielson E. “AMPK Regulates HER2/EGFR Activity in HER2-Positive Breast Cancer.” Pathology Grand Rounds. (2013) Johns Hopkins School of Medicine, Baltimore, MD.